Binding sites for glutamate and aspartate in the rat cerebral cortex during ontogeny

Thesis

BINDING SITES FOR GLUTAMATE AND ASPARTATE IN THE RAT CEREBRAL CORTEX DURING ONTOGENY

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ABSTRACT

The interaction of the putative excitatory neurotransmitters glutamate and aspartate with rat cortical membranes was examined by the utilization of a radiochemical binding assay. The specific binding of ^{3}H-L-Glu at 0^{o}C reached a maximum after approx 12 mins. and was measurable over a pH range of 4 - 9 although pH7 was optimal. The binding was strongly dependent on the presence of K^{+} ions and showed a biphasic response to the presence of Na^{+} ions, being inhibited at concentrations below 10mM but stimulated several fold at higher (Na^{+}) reaching a maximum at around 40 mM.

It was possible to distinguish two binding sites for glutamate on cortical membranes prepared from adult rats. One of these was a low density high affinity site, the other was present at higher concentrations and lower affinity and was strongly dependent on the presence of Na^{+}. Aspartate also bound to adult cortical membranes with similar characteristics. Discrimination between these glutamate and aspartate binding sites was possible as the binding of these ligands was differentially affected by freeze/thawing and by amino acid analogues investigated in displacement studies.

The binding of the excitotoxic glutamate analogue kainic acid shows characteristics incompatible with its proposed role as a glutamate agonist with affinity for the putative post-synaptic glutamate receptor site.

The development of glutamate and aspartate binding sites was also investigated. The binding of both ligands showed very different characteristics in young rats (30 days) than in adult rats. It was

much more difficult to distinguish between glutamate and aspartate binding sites in these younger animals.

The binding of glutamate to cortical membranes prepared from normal mice and 'reeler' mutant mice was also investigated in an attempt to localize the binding to a specific cortical area.

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ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
A.D.C.P.	Amino Dicarboxycyclopentate
A.M.P.A.	Amino - 3 hydroxy - 5 methyl isoxazole - 4 proprionate
ANS	Autonomic nervous system
L-Asp	L-Aspartic Acid
B MAX	Maximal number of binding sites
Ci	Curie
cGMP	cyclic Guanosine 5' phosphate
CNS	Central nervous system
D ∝ AA	D ∝ Aminoadipate
D ∝ APV	D-∝-2-Amino 5-phosphonovalerate
ED50	Dose at which 50% of maximal excitation occurs
EM	Transmembrane potential difference
EPSP's	Excitatory postsynaptic potentials
G.A.B.A.	Gamma-aminobutyric acid
G.D.E.E.	Glutamate diethyl ester
G.D.P.	Guanosine 5' diphosphate
L-Glu	L-Glutamic acid
G.T.P.	Guanosine 5' triphosphate
5-HT	5 - Hydroxytryptamine
Ibo	Ibotenic acid
IC50	Concentration at which 50% of maximal inhibition occurs
KA	Kainic acid

Kd	Dissociation constant
L.O.T.	Lateral olfactory tract
L.T.P.	Long term potentiation
m.e.p.p.	Miniature endplate potential
M.T.H.F.	Methyltetrahydrifolate
NMLA	N-methyl-L-Aspartate
NMDA	N-methyl-D-Aspartate
P.D.A.	2,3, Piperidine dicarboxylic acid
P.N.S.	Peripheral nervous system
P.S.P.'s	Post-synaptic potentials
PZDA	3, Piperazine dicarboxylic acid
Quis	Quisqualic acid
s.e.m.	standard error of the mean
S.P.M.	Synaptic plasma membranes

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CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

There are many fundamental questions concerning the functioning of the mammalian brain that still remain to be elucidated. If an answer to any of these questions is possible, it will only be through the concerted integrated application of research techniques from many different disciplines in the brain sciences. The one concept assumed in all the approaches however, is that of chemical neurotransmission as the main method by which an electrical impulse arriving at a nerve ending along an axon can succeed in affecting the next neuron in the pathway, although axon and cell body are separated by a gap of about 200-500 Å - the synapse. Because the reaction mechanism by which this occurs is so well established at those synapses using Acetylcholine and the

amines dopamine, noradrenaline and serotonin (5HT), it is perhaps easy to forget that transmission by these established transmitter substances accounts for only about 1% of all synapses in the mammalian brain. (Snyder 1973). This estimate has been arrived at mainly by treating brain slices with histochemicals which specifically interact with biochemical components, mainly enzymes of the cholinergic and catecholaminergic transmitter systems. However, a 1% sample may simply not be good enough to assume that transmission at all synapses occurs in the same way. Different chemical mediators could exert their effect by different mechanisms. The crucial requirement then is to establish the identity of the centrally acting transmitters, but although many substances have been suggested for this function, their role remains putative. However, among the strongest candidates to emerge for the role of centrally active excitatory transmitter are the dicarboxylic amino acids, glutamate and aspartate. The work reported in this thesis was initiated to help elucidate this putative function for the amino acids in the rat cerebral cortex.

The introductory chapter of the thesis first of all critically assesses the assumptions that are made concerning the mode of action of neurotransmitters because these assumptions strongly influence the choice of the experimental techniques to use in the search for novel transmitters. The question is; how applicable are these techniques when amino acid candidates are being considered as transmitters? The remainder of the chapter reviews the accumulated evidence implicating glutamate and aspartate as neurotransmitters in the C.N.S. and provides a justification for the experimental approach used throughout this thesis, namely an investigation of the interaction of glutamate and aspartate with

binding sites on brain cell membranes utilizing a radiochemical binding assay.

Much of the work is a study of the interaction of glutamate and aspartate with brain cell membranes from rats throughout their development from neonates to adult (50 days of age). Chapter 2 begins with a rationale for this line of study and goes on to consider theoretical aspects of receptors; their form, function, and relationship to other components of the physiological system they are part of. Finally, the binding assay itself is considered, in particular the problems inherent in its design and the precautions that must be considered in setting up a binding assay. Chapter 3 is a statement of the materials used and methods of preparing the tissue utilized in the assay.

Chapters 4 and 5 are a presentation of results, with Chapter 4 being a characterisation of the optimum conditions necessary for a qualitative and quantitative study of the interaction of L-glutamate with its binding sites. Each section in Chapter 5 is self sufficient, in that there is a brief introduction explaining the rationale behind each series of experiments and the possible implications of the results are discussed before moving on to the next section. There is however a logical progression from one section to another in terms of the development of ideas, although some of this logic is retrospective so that the work is not always presented in its chronological position. Chapter 6 is a summary of the results together with a critical discussion of the overall significance. The thesis finishes, of course, with ideas for future experiments that may prove worthwhile.

1.1 Peripheral Nervous System v Central Nervous System

Given that knowledge of the particular transmitter species used at any one synapse in the C.N.S. is a prerequisite for establishing the exact mechanism by which that transmitter functions, we arrive at a 'chicken and egg' situation. In trying to establish the identity of a transmitter, how do we know which biochemical parameters would be most appropriate to investigate? This paradox has been overcome by the establishment of guide-lines or criteria which must be fulfilled before a transmitter role for any substance is identified. However, these criteria are very much based on extensive experimental data concerning the mode of action of the accepted transmitter Acetylcholine, at the sites where its transmitter status was first established - terminals of the parasympathetic nervous system innervating smooth muscle and the vertebrate neuromuscular junction. Both of these sites exhibit distinct morphological characteristics and a nerve impulse initiated in the presynaptic fibre gives rise to an easily measurable physiological event, e.g. muscle contraction.

The neuromuscular junction (NMJ), in particular, provides an excellent model for neurophysiological and neurochemical analyses. A single efferent axon branches only at the point of contact with a muscle fibre to form a discrete structure, the motor end plate, composed of several structurally specialised junctions. The efferent nerve can be artificially stimulated and changes in membrane potential in the specific area served by the incoming nerve can be accurately assessed by the careful implantation of recording electrodes at the post synaptic site. In vitro preparations maintained in artificial media allow for an investigation of the ionic requirements of the mediation process and the effect of various chemical agents, administered iontophoretically, on the intensity and duration of post synaptic potentials (P.S.P's) can be estimated.

In contrast, any neurophysiological or neurochemical investigation in the brain is, inevitably, technically much more difficult. The complexity of interactions even within any one brain area, the high degree of cellular heterogeneity and the paucity of knowledge of the function subserved by the majority of neuronal pathways are daunting.

Unlike the muscle fibre any one neuron in the brain is literally covered with synaptic boutons up to a few thousand per cell. The signals impingeing on the neuron arise from many different brain areas in regions which may be far removed from the nerve ending. The cell bodies of origin may constitute specialized sensory organs, or intermediary ganglia themselves in contact with other brain regions subserving specific functions. The incoming signals are both excitatory and inhibitory and are undoubtedly mediated by several different neurotransmitter species. Besides these axosomatic interactions there are also synapses involving physiologically functional connections that are axodendritic or axoaxonic in nature. The process of synaptic transmission in the C.N.S, because of this multiplicity of interactions, is also likely to involve the participation of mechanisms for controlling or modulating synaptic activity so that the integration of many conflicting signals produces the precise physiological response demanded in a particular situation. It is not necessary to postulate that these modulatory events will occur in the locale of the synapse. Chemical modulators could exert their effect, for example, by specifically interacting with the ion channels that control the flow of charged particles across the pre- and postsynpatic membranes, rather than with the neurotransmitter receptors directly.

The application of experimentaltechniques appropriate in the peripheral nervous system (P.N.S.) to a study of the C.N.S. may then, in themselves, disrupt the delicate balance of the many integrative processes

involved in the function of a discrete brain area, let alone a single neuron. This in turn may make the process of extrapolation from experimental data to events in vivo a little more uncertain.

Thus a consideration of the structural differences between the central and peripheral nervous system is alone sufficient, in my belief, to warrant caution in the application of the criteria for neurotransmitter status. Further criticisms based on the doubtful universality of the mechanism by which acetylcholine exerts its effect (Werman 1966) will be relevant in later sections where I wish to argue that acceptance of the criteria may have had a retrogressive and damaging effect on research into chemical transmission in the C.N.S. However, as the work reported here was initially formulated and carried out within the framework demanded by a particular paradigm, I think it is important to consider from a historical perspective the scientific climate within which the criteria were developed.

This allows for the assessment of the validity and usefulness of establishing the criteria in the first place, and an understanding of how they may have achieved their sacrosanct status. It may also serve to justify their continued application, albeit within the context of a more critical approach.

1.2 AN HISTORICAL PERSPECTIVE

Despite the morphological simplicity apparent within the autonomic and peripheral nervous systems, and the targets they innervate, there were still enormous problems in establishing the existence of chemical transmission, let alone the identity of the transmitters acetylcholine and noradrenaline. I think it is possible to isolate two main reasons for these problems. First of all, when the concept was first suggested early this century there were none of the technical aids now taken for granted such as sensitive intracellular electrical recording instruments and the electron microscope. Techniques were based mainly on those established by classic physiology - the perfusion of isolated tissue systems and organs in vitro and the effect on their responses of a wide range of experimental manipulations. Perhaps the underlying difficulty though, was the need to overcome the dogmatism of scientists at that time who rigorously held the view that nervous activity was purely an electrical phenomenon. For a quarter of a century there was considerable prejudice against the concept of chemical transmission even in the face of rapidly accumulating evidence.

Unfortunately it is arguable that winning that particular battle may have simply resulted in the replacement of one dogmatic conceptual approach with another, which in its turn has slowed down the investigation of the many other chemical substances and modes of action used by the nervous system to transmit messages.

One of the first discoveries of electric transmission came from Dubois Reymond in 1843. At that time it was thought that nervous tissue was a continuous sheet of connected cells, the nerve impulse being propagated from cell to cell by uninterrupted flow of current

through a continuous medium. Nevertheless, in 1877 Du Bois Reymond himself tentatively suggested that chemical molecules may also be involved at some stage. At around this same time the brilliant Spanish histologist Raman y Cajal was making a series of meticulous and perceptive drawings of many different cell types. His study of nervous tissue revealed that at the point of contact between nerve cells there was a small gap. Thus the cell theory was born, nervous tissue was not uniformly continuous but composed of a network of individual units. Nevertheless the concept that transmission of the nerve impulse across this gap may be mediated by chemicals was still discounted as it was mistakenly assumed that the process of diffusion would be too slow to account for the known high speeds of impulse transfer in the C.N.S. In retrospect this seeming short-sightedness can be explained by the fact that although Cajals work had revealed the presence of intercellular spaces, the limits of resolution of microscopes at that time were not sufficient to reveal the structural specialization of the synapse responsible for aligning pre-and post synaptic membranes in close delineated apposition. The effects of nervous stimulation on glandular secretion presented no such problem. Both the time of onset of the physiological response and the duration occurred over time scales considerably greater in magnitude than the stimulation of motor activity.

A significant advance came in 1904 when Eliot discovered that an adrenal gland extract, adrenaline, could produce the same effects as sympathetic nerve stimulation. This line of reasoning was followed in 1907 by Dixon who electrically stimulated a perfused frog heart and collected the perfusate during and immediately following stimulation.

He re-applied the perfusate directly onto the heart but failed to evoke any change in muscle contraction. The definitive difference between the two experiments is again only apparent in retrospect. Acetylcholine is degraded by acetylcholinesterase quickly following its production and secretion into the synaptic cleft.

Dixons failures unfortunately discouraged further endeavours in this direction for many years. Serendipity played a part in 1921 when Otto Loewi linked two frog hearts in individual organ baths by perfusing them with the same medium flowing in through the first heart and out through the second. Electrical stimulation of the first heart was followed after a short time delay by a slowing of the heartbeat in the second which Loewi interpreted as being due to a chemical agent liberated into the perfusing medium from the first heart (see Loewi 1940). Although the experimental design and execution were faultless given technical facilities available at that time, Loewi too may have failed to provoke stimulation of the 2nd heart if he had not, unknowingly, taken advantage of a seasonal variation in the ratio of Acetylcholine to its degradatory enzyme acetyl-cholinesterase in frogs which during the time he carried out his experiment, (Easter) was at its maximum! (Bacq 1975).

Subsequent attempts to emulate Loewi's work over the next five years only suceeded in arousing controversy. The publication of widely differing data using similar experimental operations alternated in providing the opposing lobbies (for and against chemical transmission) with ammunition! Historical retrospectives on this period from scientists eminent in the debate (Bacq,1975 ; Dale,1953) reveal an intense polarization of viewpoints with continuous heated and bitter debates.

The tide began to turn in the favour of the theory of chemical transmission when routine screening of a variety of compounds for their

ability to emulate or antagonize the effects of stimulation by the sympathetic nervous system revealed the acetylcholine analogue eserine to be a potent enhancer of the physiological effects of ACh on glandular secretion and smooth muscle contraction. The inclusion of eserine in the perfusing medium of subsequent experiments allowed the development of highly reproducible experimental methods and led to the correct conclusion that eserine exerted its effect by inhibiting a synastically active degradatory enzyme for acetylcholine. (See Loewi 1940).

Eserine was subsequently used unquestioningly in all investigations. At the motor end plate however, this simply led to massive depolarization and tetani rather than the smooth graded contraction observed in the absence of eserine. This was interpreted critically as evidence that eserine had been exerting its effect by acting directly as a toxin and not by enhancing synaptic concentrations of Acetylcholine. Application of ACh in the absence of its esterase however (i.e. on denervated muscle) did mimic motor nerve stimulation, and a series of experiments in the 1930's by Dale and his colleagues culminated in the collection and identification of Acetylcholine as the substance released from motor nerves on stimulation (Dale 1938).

Research into the nervous system had thus evolved a new branch, that of neurochemistry, which even during its infancy began to challenge the dominance of the physiological approach. The two disciplines were unfortunately seen by many as noncomplementary under the strong influence of the leading electrophysiologist of that period, Sir John Eccles, a formidable opponent of chemical transmission as an explanation of neuronal communication and a strong adherent of explanations in terms

of electrical phenomena only.

The swelling tide of technological advancement was against him and the advent of the elect on microscope, sophisticated intra-cellular recording techniques together with iontophoresis, and histochemical techniques all served to confirm the validity of the concept. For the first time the complexities of synaptic structure could be seen and specific labelling and staining of components of the cholinergic and catecholaminergic systems allowed for the localization of storage vesicles and metabolizing enzymes to nerve endings.

Eccles admitted defeat with grace and integrity in 1945, albeit with the guidance of the Scientific philosopher Kark Popper. His conversion was complete and dramatic (Eccles, 1970) to the extent that he became almost overnight one of the most ardent exponents of the concept of Chemical transmission. In the following years he relentlessly applied his electrophysiological expertise to the study of the mechanisms of chemical synaptic transmission and became one of the most brilliant and well respected experimenters in this field. His work led to the winning of the Nobel Prize in 1963, in particular for his microelectrode studies of synaptic transmission in the motor neuron reflex system (Eccles 1966, 1964).

The demonstration of the validity of the concept of chemical transmission and the identification of ACh and the catecholamines as mediators was arguably one of the major advancements of scientific thinking to occur this century. For the first time, brain activity could be described not only in terms of electrical events, but also in terms of distinct specific chemical interactions at the exact site of communication between two neurons, the synapse. The regulation of

synaptic activity could now be explained in terms of different signals, excitatory and inhibitory impingeing on a cell and mediated by different messengers. The advancement, however, was hard won and, with the benefit of retrospection it is possible to see the development of the criteria as a protection mechanism to shield and defend the birth of a new concept in an alien environment.

The original experiments of Eliot, Loewi and Dale were quickly followed with others by enthusiastic, though less competent workers, leading to claims of synaptic involvement for many substances with little or no regard for attempts to control for other explanations in the data. The establishment of a coherent scientific methodology for neurochemical research was thus seen as essential. Critical objections to many investigations only aided the unquestioning acceptance of the need for the fulfullment of certain criteria as a prerequesite for establishing a role for a substance as a neurotransmitter. The criteria, initially intended as guidelines of approach, assumed the status of rules of investigation and became sacrosanct. The criteria applicable for studies on the CNS were originally a simple extension of those utilized for peripheral transmitters (Paton, 1958). Although extensive criticism (Werman, 1966; Johnson, 1971; Orrego, 1979) has led to their partial modification, in their present form they unquestionably reflect the wealth of knowledge accumulated concerning the mode of action of acetylcholine in the autonomic and peripheral nervous systems.

1.3 CRITERIA FOR IDENTIFICATION OF NEUROTRANSMITTERS

Perhaps the most fundamental of the criteria is that of 'Identity of Action' which states that when a transmitter-suspect is applied in the synapse it should produce in every respect the same postsynaptic effect as the natural transmitter as measured by neurophysiological (e.g. the generation of PSP's) and neurochemical (e.g. change in ion movements and in 2nd messenger levels) techniques. A corollary to this is that of 'collectibility'. A putative transmitter substance collected upon stimulation of the presynaptic neuron and used to stimulate the post-synaptic cell with identical results as natural stimulation, offers the most comprehensive fulfillment of this criterion.

An extension of the Identity of Action criterion is that of 'Pharmacological specificity'. The postsynaptic effects of a putative transmitter substance should be mimicked by drug agonists and modifiable by antagonist in the same manner as the natural transmitter reflecting the structure activity relationship of the transmitter to its receptor on the postsynaptic cell membrane.

A third criterion is that of 'induced release'. The candidate should be selectively released from presynaptic nerve endings upon depolarization of the nerve by physiological means. It must be released in amounts necessary to cause the known physiological effect on the postsynaptic cell and directly proportional to the magnitude of stimulation. The release process must also show a dependancy on extracellular calcium concentration because influx of Ca^{++} ions into the presynaptic nerve terminal upon depolarization has been suggested as necessary for release by a process of stimulus-release-coupling (Katz, 1969). Evidence for this involvement of

calcium on the release process was obtained by Katz and Miledi (1970) and Miledi (1973) in studies on the release of Acetylcholine from the squid giant axon.

The above criterion is essentially presynaptic and follows on from criteria that state that the candidate must be present in nerve endings thought to use the substance as their transmitter, together with metabolic enzymes involved in maintaining a transmitter pool from readily available precursors.

The balanced physiological effect of transmitters is dependent on their rapid removal from the synaptic cleft on cessation of the depolarizing impulse causing their release. Thus a primary criterion is the demonstration of some mechanism for rapid termination of the transmitter function of the candidate.

A final criterion demands that neurotransmitter candidates should be differentially distributed throughout the CNS, so that their varying concentrations in different brain regions should parallel the functional specification of different projection pathways.

From such considerations the transmitter status of a select group of substances has been established as shown in Table 1 according to Iversen (1980). Table 2 shows the suggested transmitters together with the possible pathways whose activity they mediate.

Accepting the proposition that there is a multiplicity of transmitter species utilized in the CNS (Krnjevic 1974), and that the criteria are a relevant reflection of their mode of operation, it may be expected that following four decades of active research Table 1 should by now include many of the candidates in Table 2. That this is not so points I believe

TABLE 1 ACCEPTED NEUROTRANSMITTERS

Amino Acids	Amines
Gamma-aminobutyrate, GABA	Acetylcholine
Glycine	Dopamine
	Noradrenaline
	Adrenaline
	5 Hydroxytryptamine

TABLE 2 PUTATIVE NEUROTRANSMITTERS

Amino Acids	Others	
Glutamate	Corticotropin	PITUITARY PEPTIDES
Aspartate	Growth hormone	
Proline	Lipotropin	
Taurine	∝ Melanocyte stimulating hormone (MSH)	
	Oxytocin	
	Vasopressin	
Adenosine		
	Angiotensin	CIRCULATING HORMONES
	Calcitonin	
	Glucagon	
	Insulin	
	Cholecystokinin CCK	GUT HORMONES
	Gastrin	
	Motilin	
	Pancreatic polypeptide (PP)	
	Secretin	
	Substance P	
	Vasoactive intestinal polypeptide (VIP)	
	Dynorphin	OPIOID PEPTIDES
	β-endorphin	
	Met-enkephalin	
	Leu-enkephalin	
	Kyotorphin	
	Corticotropin-releasing factor (CRF)	HYPOTHALAMIC RELEASING HORMONES
	Luteinizing-hormone-releasing hormone (LHRH)	
	Somatostatin	
	Thyrotropin-releasing hormone TRH	
	Bombesin	OTHER PEPTIDES
	Bradykinin	
	Carnosine	
	Neuropeptide Y	
	Substance K	
	Prolactin	

to the need for a major reappraisal of the mechanisms and parameters constituting chemical neurotransmission and a reassessment of the criteria, acknowledging the possibility that, in many ways, acetylcholine may be the exception to the rules governing the activity of transmitters, if there are rules at all.

1.4 CRITIQUE OF NEUROTRANSMITTER CRITERIA

All of the criteria are open to misinterpretation due partly to the limitations of the techniques employed in their study, some of which warrant further discussion, but also due to the particular conceptual approach adhered to by each individual researcher. I can achieve no better summation of this than a statement by Orrego (1979).

> "More dangerous than the inherent defects of the criteria, however, seems to be their injudicious application, usually because of an overzealous wish to demonstrate that one's favourite molecule is indeed a transmitter."

Orrego (1979) also correctly emphasized the interdependency of the criteria. Their strength can only be in their conjunction, the demonstration of each being in itself insufficient proof of transmitter function. This requirement is essential even for those candidates whose locations and proposed function is limited to nervous tissue. This is precisely because the unique set of problems defining the limitations of each criterion must preclude an accurate assessment of their relevance to prove or disprove a transmitter role.

How much more limited then must the criteria be in clarifying such a role for substances whose ubiquity throughout the CNS as well as the rest of the body is a reflection of a central role in metabolism, quite apart from any proposed synaptic activity. The dicarboxylic amino acids glutamate and aspartate emerged as transmitter candidates following a series of studies by Hayashi (1952, 1954) on the causation and possible treatment of epilepsy. Among other naturally occurring brain constituents L-Glutamate (L-Glu) and L-Aspartate (L-Asp) were found to produce convulsions in apes and dogs, upon injection directly into the cortex, although the experiments could not determine either the site or mode of

action of the convulsants.

The development of the technique of microelectrophoresis in the late 1950's was subsequently exploited by Curtis et al (1959, 1960, 1963, 1965) in a search for new transmitter candidates. Their studies revealed a series of neuronal excitants endogenous to brain tissue essentially similar to those identified as convulsants by Hayashi and dominated by L-Glu and L-Asp. In vivo intracellular recording demonstrated that the excitatory action of L-Glu was a result of a depolarization of the neuronal cell membrane showing many characteristics of naturally occurring EPSP's. Significantly, the PSP's differed in some respects from those evoked at cholinergic synapses - an inevitable signal for scepticism and caution.

The primary criterion of identity of action (Werman, 1966; Orrego, 1979) demands, as neurophysiological evidence, just such a demonstration that post-synaptic potentials evoked by iontophoretically administered candidates are identical to those produced upon stimulation of the presynaptic nerve. In vivo studies in the C.N.S. utilize micropipettes closely aligned to the somatic membrane of a susceptible neuron. The technique presupposes that in keeping with the mode of action of ACh in the P.N.S., the perikaryal membrane is the transmitter receptive component. Even if this were indeed the case, neuronal soma in the C.N.S. possess innumerable postsynaptic sites undoubtedly receptive to many different transmitter species. Although the technique allows for an accurate estimate of the amount of any substance released from the pipette, the complexity of cellular inter-relationships denies any knowledge of the size of the intercellular spaces available for the diffusion of the substance and thus its effective concentration at the relevant synapse. Extremely large local concentrations of a non-transmitter substance may thus lead to

E.P.S.P.'s by virtue of the applied substance being a weak agonist of the true transmitter. Conversely, very low concentrations of an applied candidate in the synaptic cleft may be insufficient to raise the membrane potential above the threshold for depolarization even though it is indeed the transmitter. Low levels of injected substances in the vicinity of the synapse could arise not only as a result of passive dilution and diffusion effects, but also by active removal of the substance either by enzymic action or by uptake into the surrounding cells. This is a very real problem with candidates such as the amino acids that serve a general metabolic function as uptake mechanisms designed to accumulate the amino acids for these functions may also serve to remove the chemical artificially introduced into the intercellular space. There also remains the possibility that an injected substance may cause the release of the natural transmitter by its effect on the presynaptic nerve ending leading to a false estimate of its transmitter role. These problems may be circumvented to some extent during investigations of the effect of ACh in different brain regions. First of all, its synaptic activity is known to be terminated by a specific clearing enzyme, AChE, which can be inhibited specifically during experiments. Second, iontophoretically administered ACh has been shown to inhibit its own release, so that levels of ACh in the synaptic gap are likely to be uncomplicated by either excessive degradation or enhancement by presynaptic release. This mechanism of auto-inhibition has not been demonstrated as yet for Glutamate and Aspartate. In addition, difficulties encountered in the study of the excitatory effects of these amino acids are exacerbated by the fact that excitatory synapses are likely to be located on dendrites far removed from the cell body (Szentagothai, 1971). Evidence for this comes from studies correlating the potency and time course of the action of iontophoretically applied amino acids with the degree of dendritic arborization (Herz, et al 1969; Schwartzkroin and Anderson, 1975; Zieglgansberger and Champagnot, 1978).

The collection, identification and subsequent application to post-synaptic cells of substances released from nerve endings, with the demonstration of identical physiological responses as those evoked by natural transmission, would indeed represent the most conclusive fulfillment of the 'identity of action' criterion. Although such studies directly established the role of ACh in the ANS the practical relevance of such an approach to investigations in the CNS must be seriously questioned. The technical problems inherent in the location and isolation of the functional nerve in vivo, and in the design of the collecting apparatus, would seem at the present time to pose insurmountable problems. The conceptual basis for collectability is also suspect as it requires first of all that the transmitter should be produced and released in vast excess of amounts needed to evoke the physiological response. If this were not so the possibility must exist that concentrations of the transmitter in the intercellular fluid may be too low for detection, despite the recent development of sophisticated analytical techniques. Secondly, the maintenance of measurable amounts of transmitter in the synaptic cleft requires at least a partial blockade of the method or methods normally used to terminate the activity of the transmitter after it has served its mediatory function. Again, studies of the transmitter action of ACh utilize specific competitive inhibitors of the degradatory enzyme AChE, greatly enhancing synaptic concentrations. Unfortunately, ACh seems to be the only transmitter studied so far whose synaptic activity is terminated by enzyme action. The catecholamines and the inhibitory amino acid transmitter Gamma-amino butyrate (GABA) are removed from the synaptic cleft by rapid high affinity reuptake into the presynaptic ending or into the surrounding cells. If this were true for putative transmitters then specific blockers of such uptake would need to be utilized, paradoxically demanding prior knowledge of the structural requirements of the proposed reuptake system!

Based on the assumption that the postsynaptic physiological effect is evoked by a series of events initiated by the interaction of a transmitter with a specific membrane bound receptor, that effect should be capable of being specifically blocked (antagonized) or mimicked by agents with structural similarities to the transmitter. Thus the iontophoretic application of such a drug should have identical effects to the interaction of the natural transmitter with the postsynaptic membrane and the suspected transmitter also exogenously applied. A prerequisite for this criterion is that the pharmacological agent should be strictly discriminatory for the population of receptors specific for the putative transmitter, as opposed to those for other transmitters impingeing on the cell. The likelihood of an agent having this property will be greater if the transmitter has an unusual chemical structure unrelated to other naturally occurring brain constituents. Thus, specific nicotinic and muscarinic cholinergic antagonists have been widely used to confirm the transmitter function of ACh at specific sites, such as the synapse between spinal motorneurons and Renshaw cells (Eccles et al, 1956).

Problems arise, however, when the transmitter function of the excitant amino acids is being considered because pharmacological agents so far discovered seem limited in their ability to discriminate between populations of receptors for Glutamate and Aspartate (Watkins, 1978). Thus in this case it is necessary to test the antagonistic efficacy of a long series of structurally related compounds and hope for differences in the graded potency of their effect on the synaptic action of the two candidates. If this same series of compounds antagonized the effect of the natural transmitter in the same order, then some discrimination between the site of action of the two putative transmitters may be possible. Absolute identification of a transmitter using this criterion however, is unlikely to occur as considerable overlap in the effects of many of the agents must

be suspected. Nevertheless, it may be possible by this approach to reach an understanding of the strict structural requirements of the receptor sites which may in turn lead to the development of synthetic discriminatory analogues. Thus even with candidates as structurally similar as glutamate and Aspartate, this line of approach would seem to be one of the most fruitful in the pursuit of criteria fulfillment.

Any estimation of the synaptic role of candidates that are important in general metabolism must seek to discriminate between a neurotransmitter function and biochemical parameters relating to their ubiquitous role in chemical homeostasis. Thus there is the danger that release studies in vivo, or using tissue slices in vitro, may fail to distinguish between release of the amino acids at synaptic junctions and that occurring from other compartments, perhaps as a result of generalized changes in membrane permeability of neuronal and non-neuronal elements during electrical activity (Matsui and Yamamoto, 1975; Nadler et al, 1977). Aspartate and Glutamate both show a resting release from all neural tissue so far studied and the amount of release can be raised by the application of extracellularly applied K^+ (50 mM) or electric field stimulation designed to mimic or evoke action potential and thus pseudo-physiological in nature. Thus, the criterion of induced release alone would seem to be inapplicable to a study of the synaptic function of the amino acids, unless it is possible to show regional variation in the extracellular levels of candidates following the release process. Any such regional variation must correspond to areas highly innervated with glutamatergic or aspartergic projections and must therefore reflect regional variations in intracellular concentrations of the amino acids. Even if release did not show patterns classically suggestive of a transmitter role this does not preclude the possibility that both amino acids could fulfill a much more generalized function in the CNS as well as any specific transmitter role. From a

consideration of their universal excitatory effect on neurons, this general role, speculatively, could be to enhance effectiveness of natural neurotransmitters by maintaining postsynaptic membranes just below the threshold essential for depolarization.

The dependancy of the neurotransmitter release process on extracellular Ca^{++} levels is well documented and has been summarized in recent reviews (Llinas, 1977; Llinas and Heuser, 1977; Rahamimoff, 1976). Thus, the criterion demands that the release mechanism should be calcium requiring. Evidence for this involvement of Ca^{++} was first obtained by Katz and Miledi (1970), using the cholinergic squid giant axon. A recent review by Kelly (1979) emphasises that the exact mechanism by which neurotransmitter release occurs is not well established, but all theories are based on investigations within the experimental paradigm of the quantal theory proposed by Katz and coworkers in the early 1950's (Fatt and Katz, 1952; Del Castillo and Katz, 1956) and developed into the vesicle hypothesis following the description of densely packed vesicles in the presynaptic nerve terminal (DeRobertis and Bennett, 1954, 1955). The quantal theory was developed to explain the generation of miniature endplate potentials (mepp's) of constant amplitude at the unstimulated NMJ. Each mepp was hypothesised as due to the spontaneous release of the contents of one synaptic vesicle into the synaptic cleft whilst simultaneous discharge of many vesicles in response to the arrival of a nerve impulse would generate the endplate potential. Thus, within this paradigm the role of Ca^{++} is assumed to be due to the need to overcome an energy barrier, which, under resting conditions, serves to prevent apposition of synaptic vesicles containing transmitter with the cytoplasmic surface of the perikaryal membrane. Fusion of the membranes following Ca^{++} influx through voltage sensitive channels would be followed by exocytosis of vesicular contents into the synaptic cleft. The energy barrier itself

has been postulated to be due to either electrostatic repulsion between the membranes (Dean, 1975) or to a hydration barrier (LeNeveu, et al, 1977). The theory of Katz (1969) and Berl et al. (1973) that the role of Ca^{++} could be in a stimulus release coupling process has already been mentioned, whilst other workers have suggested that Ca^{++} exerts its effect by screening of negatively charged phospholipid components leading to a cross bridging between vesicle and perikaryal membranes.

Unfortunately, however, experimental data supporting the vesicular hypothesis is equivocal, even for Ach at the NMJ, and there is considerable evidence to show that even though the vesicular hypothesis may be valid at certain synapses, it may not be for others. Thus it has been shown that in the electric organ of Torpedo, ACh was preferentially released from a cytoplasmic pool (which constituted at least 50% of the total ACh) as opposed to a vesicular pool during stimulation <u>in vivo</u> and <u>in vitro</u> (Dunant et al, 1972). The explanation of the role of Ca^{++} in such a non-exocytotic scheme is more difficult but could be to activate carrier proteins or membrane gates to allow the outward passage of ACh. The function of the vesicles could be as a store of ACh replenishing cytoplasmic levels as required, or in the sequestration and ejection of intracellular calcium. If activation of carriers or opening of "gates" are the methods by which Ca^{++} exerts its effects, it is possible if not likely, that carriers or channels mediating the transfer of different transmitters out of the nerve ending may have differing requirements for Ca^{++}.

Thus, investigations of specific release can only be of limited value in providing proof of transmitter status, as long as the conceptual basis underlying these investigations is suspect. Significant to this argument is the fact that none of the amino acid candidates has been found in

synaptic vesicles prepared from cerebral cortex after purification by gel filtration (Rassin, 1972; DeBelleroche and Bradford, 1973). It is therefore arguable from the above considerations that before specific release can be justified as an absolute criterion for neurotransmission, considerable effort should be devoted to a clarification of the mechanisms mediating release in different types of neural tissue and at different synapses. Considerable depth of knowledge concerning the constitution of possible storage complexes within the nerve ending is also essential, as release of a substance into the synaptic cleft upon physiological stimulation may occur because that substance is packaged and released with the natural transmitter, acting postsynaptically in a modulatory capacity although having no transmitter function of its own.

Werman (1966) also pointed to the possibility that more than one transmitter substance may be released from one presynaptic terminal. Thus two transmitters working synergistically may be necessary to evoke P.S.P.'s. In this case the collection and purification of a single substance, specifically released from nerve terminals and re-applied to the postsynaptic membrane, would not mimic the physiological event exactly, leading to a false appraisal of its role. This is especially interesting in view of several reports stating that glutamate and aspartate are both released from certain brain areas upon stimulation and that they have a synergistic effect when applied iontophoretically at the invertebrate NMJ (McBurney and Crawford, 1979).

The presynaptic criteria demanding localization of transmitter candidates, and their metabolizing enzymes, to nerve endings utilizing them as transmitters has obvious limitations when substances are considered with functional roles in general metabolism as well as any transmitter function. Thus for Ach and the catecholamines it is possible to show

restricted location associated with discrete brain areas. This cannot be the case for the amino acid candidates whose ubiquity throughout the CNS is a reflection of their general metabolic role. Thus the task presented by this criterion is not to show presence or absence of components of the proposed transmitter system in certain brain areas, but to show a quantitative differential distribution and to demonstrate conclusively that such differences are due to transmitter excess over general metabolic needs. Given that there are concentration gradients across brain regions for all CNS constituents, even water, (Dickerson, 1968) there follows the added problem that minor regional variations may not be significant in ascribing a transmitter role and that a subjective estimate as to the size of the difference that constitutes significance must be made (Orrego, 1979). The same author also correctly pointed out that there may be transmitters that are ubiquitous simply because they mediate the important function of central excitatory (or inhibitory) transmitters throughout the CNS.

Potentially one of the most useful of the criteria in establishing a transmitter role for a candidate is the demonstration that there exists a method of inactivation capable of rapid removal of the transmitter from the synaptic cleft on cessation of the nerve impulse. This is an essential requirement as the mode of action of transmitters is to temporarily disrupt ionic balance between the inside and outside of the postsynaptic cell allowing for hyper-or depolarization of the cell membrane. If such ionic disturbances were allowed to continue for long periods many cellular processes relying on the maintenance of osmolarity and local ionic changes within very narrow limits may be irreversibly disrupted, leading to cell damage or death.

The inadequacies of this criterion would seem to lie in the consideration of too narrow a range of potential mechanisms capable of fulfilling this role on the one hand, and a lack of consideration of alternative functions for such mechanisms on the other. Based on the cholinergic and catecholaminergic systems, the presence of a degradative enzyme for the transmitter in the synaptic cleft (analogous to AChE), or the presence of a high affinity reuptake system, are considered adequate for fulfillment of this criterion.

Problems of interpreting the role of inactivating mechanisms again only arises when a substance is considered which has other functions in the CNS. Thus, the demonstration of a catabolic enzyme does not necessarily mean that the enzyme is used to terminate transmitter activity. Also, the presence of a high affinity reuptake mechanism for the excitant amino acids could be due to the necessity for maintaining normal extracellular concentrations of glutamate or aspartate below excitotoxic levels, given the almost universal excitatory effect of these compounds on nerve cell membranes.

Werman (1966) presents evidence for several different inactivation methods and it seems plausible, if not likely, that given a heterogeneity of transmitter species in the CNS there will also be a heterogeneity of mechanisms for terminating their activity.

So this then is the critical framework within which the search for new transmitter species must be made and within which experimental data relating to such a function must be analysed. If one of the aims of neurochemical research is to reach an understanding of the processes subserving higher mental functions, then the most challenging aspect of this research is to establish the identity of the transmitters relaying information to, and within, the cerebral cortex.

1.5 EVIDENCE FOR GLUTAMATE AND ASPARTATE AS NEUROTRANSMITTERS IN THE CEREBRAL CORTEX

The dicarboxylic amino acids in general, and glutamate in particular, emerge as very strong candidates for the role of general excitatory neurotransmitter. The evidence relating to this role has been considered in several excellent reviews (Curtis and Johnston, 1974; Davidson, 1976; Krnjevic, 1974; Johnson, 1978) with much of the suggestive evidence relating specifically to their putative function in the cortex (Crawford and Curtis, 1964; Krnjević, 1965; Krnjević and Phillis, 1963). The finding discussed previously that glutamate depolarizes nerve cell membranes when applied iontophoretically, with similar E.P.S.P's as those produced by known transmitters, is crucial to this argument. This excitatory effect of glutamate is apparent on all brain neurons so far tested, and although this universality of effect has been suggested as being non-compatible with a neurotransmitter role (Curtis, 1965; Curtis and Watkin, 1963), more detailed analysis has shown a wide variation in responsiveness among neuronal populations, some units requiring much higher concentrations of glutamate for depolarization than others (Johnson, 1972). The excitatory effect of glutamate is also restricted to the CNS as it is ineffective in depolarizing neuronal elements in the mammalian A.N.S. and P.N.S. (Obata, 1974). Considered in its entirety the data are totally in keeping with a role for glutamate as the major excitatory transmitter in the C.N.S.

It has been known for some time that glutamate is much more concentrated in the brain than in all other body tissues. Whilst this is also true of other amino acid transmitter candidates it is not true for non-transmitter amino acids. Thus the brain/serum concentration ratio for GABA is 150, for glutamate and aspartate 300 and for all other amino acids

1. Potentially more indicative of a neurotransmitter role for Glutamate in the cortex is the fact that there is a stepwise increase in Glutamate concentration

from "lower" brain areas to "higher" brain areas ranging from 4.3μ moles/g in the dorsal root ganglia (where a transmitter function for Glutamate is not envisaged) to 12.4μ moles in some cortical areas of the rat brain (Johnson and Aprison, 1971). This distribution pattern is unique for glutamate among the amino acids, and although this gradient has been correlated with a sigmoidal increase in O_2 consumption and thus increasing levels of metabolic activity, it may also be representative of a greater transmitter excess over and above metabolic needs (Johnson, 1978).

A fundamental concept in the investigation of the proposed glutamate transmitter system is the discrimination between a metabolic pool of glutamate common to all neurons, and a transmitter pool present only in those neurons utilizing the amino acid as a mediator of synaptic activity. The problem of identifying different pools of glutamate is primary to an understanding of how metabolic and transmitter levels of glutamate may be differentially regulated. The considerable amount of data concerning the problem of compartmentation of glutamate in the C.N.S. is a reflection of the firm necessity for an understanding of the complexities of glutamate metabolism, prior to any discussion of a possible synaptic role.

Thus, several groups of workers have shown that glutamate metabolism in the brain is compartmentalized into two major distinct pools and that the brain is unique in this respect compared to non-neuronal tissue, (Berl et al, 1961, 1962, 1970; Van den Berg et al, 1969; Balàzs and Cremer, 1973; Benjamin and Quastel, 1974; Van den Berg and Ronda, 1976a and 1976b). The data suggest a large glutamate compartment associated with high activity of the enzyme glutamate dehydrogenase which would ensure the anaplerotic use of the available glutamate for the maintenance of an active T.C.A. energy cycle. The precursor for the glutamate is this large pool, which is essentially located in nerve endings, is glucose. A small glutamate

pool, on the other hand, is fed by exogenously supplied glutamate, aspartate, butyrate, acetate, CO_2 and other compounds which feed into a T.C.A. cycle at the level of Acetyl CoA with a reduced glycolytic involvement. Transaminases and glutamate synthetase are important to the activity of this small glutamate pool, which is characterized by a rapid turnover of glutamate to glutamine, and which seems to be predominantly situated in glial tissue. Although this overview represents a simplification of what is undoubtedly a complex phenomenon it does suggest a cycle of events involved in the putative transmitter function of glutamate separate from its general metabolic involvement. Glutamate released from nerve endings, either specifically following the arrival of a nerve impulse or spontaneously following generalized changes in membrane permeability upon activity, would be effectively removed from the synaptic cleft by the high affinity reuptake systems present mainly in glial compartments (Henn 1976). Glutamate entering neurons by a low affinity passive transport system would be reutilized for metabolic purposes. Glutamate accumulated into the glial elements on the other hand would be rapidly converted to glutamine which may function as a store for synaptically active neuronal glutamate. Large levels of glutamate released from the large pool during continuous excitatory stimulation may cause a serious depletion of T.C.A. cycle intermediates and thus a breakdown of the energy generating reactions of the cell. If on the other hand the glutamate released during nervous activity came from a rapid conversion of glutamine to glutamate catalyzed by glutaminase then levels of glutamate need not fall below those demanded for continual replenishment of T.C.A. cycle reactants. Although neurons do not possess a high affinity uptake system for glutamine (Roberts and Keen, 1974) it has been shown to pass across synaptosomal membranes very easily by passive diffusion given a glial-neuron concentration gradient (deBelleroche and Bradford, 1972; Wellbourne, 1974). Although possible differences in glutamate compartmentation on a regional basis have not yet been analyzed

and would indeed be difficult to assess, the contribution of metabolic studies to an understanding of the role of the excitant amino acids in the CNS cannot be over estimated.

One of the most important applications of data concerning metabolic compartmentation of glutamate in the brain is to the understanding of studies designed to show specific release of the amino acid from nerve endings thought to utilize it as a neurotransmitter. Before such studies can provide supportive evidence for a putative transmitter role for glutamate it is essential to show that any release of amino acid is from a specific transmitter pool, and does not represent a much more generalized release mechanism unrelated to transmitter function and which is perhaps part of a homeostatic mechanism designed to maintain extracellular and intracellular levels constant (Cox and Bradford 1978).

There have been many reports of a continuous resting release of Glutamate and Aspartate from all types of nerve tissue including those representative of non-synaptic tissue. The release, obviously unrelated to a direct transmitter function, can be increased by the application of levels of K^+ sufficient to evoke PSP's in vivo and therefore physiological in nature. (Roberts 1974; Weinreich and Hammerschlag, 1975). This is presumably as a result of generalized changes in membrane permeability during electrical activity. These in vivo findings are relevant to in vitro studies which predominantly utilize perfused tissue slices which were necessarily composed of a heterogeneity of cell and tissue types.

Although it has been stressed that a strict dependency for Ca^{++} of the transmitter release process must be demonstrated (Orrego, 1979), the non-specific release described above has also been shown to be markedly decreased as Ca^{++} levels are lowered (Roberts, 1974; Nadler et al, 1977;

Minchin and Iversen, 1974). These findings strengthen the preceeding argument (see p. 23) that although a role for Ca^{++} in synaptic release is probably indisputable the mechanism of its action at different types of synapse, its function at non-synaptic locations, and therefore the relevance of its use to distinguish between specific and non-specific release remain to be elucidated. Thus it has been stated by Johnson, (1979) that the most that can be demanded is that specific release should be completely abolished by zero Ca^{++}.

Thus the problem in release studies is to estimate any specific synaptic release over and above the appreciable levels of the amino acids already present in the extracellular space or accumulating there by other release processes. Thus levels of amino acids released at the synapse as a result of neural activity may contribute a very small proportion of the total extracellular levels (Matsui and Yamamoto, 1975; Nadler et al, 1977). Preloading of the tissue with radiolabelled amino acids and subsequent measurement of radioactivity in the medium following application of the release stimulus has attempted to overcome this problem. However, it is still difficult to interpret data from these studies as comprising transmitter release because of the possibility that the isotope could be accumulated and released by compartments other than the nerve ending (Watkins 1972). As already stated, glial cells possess transport mechanisms for the accumulation of amino acids and can be depolarized by high concentrations of K^{+} or by electrical stimulation; thus they may significantly contribute to increased efflux of labelled amino acids during release studies - although this efflux is not Ca^{++} dependent. Even if exogenous radiolabelled amino acids are accumulated into pre-synaptic nerve endings the possibility remains that they may selectively enter a metabolic pool of non-releasable compounds and that the amino acids released upon stimulation will be from a separate non-labelled pool leading to a failure to detect release.

There have been attempts to obviate these problems in radically different ways. As previously discussed metabolic studies have shown that glutamate compartmentation in the brain is segregated into a small (glial) pool and a larger predominantly neuronally located pool which is selectively fed by glucose. Potashner (1976a) exploited this finding by using ^{14}C labelled glucose as a precursor of endogenous amino acids in guinea pig cerebral cortex slices and was able to demonstrate specific evoked release of glutamate and aspartate (and no other amino acids) that was drastically decreased in Ca^{++} free medium (Potashner, 1976b).

Although undoubtedly labelling a neuronal pool of glutamate as opposed to a glial pool, labelled glucose may nevertheless only partially contribute to the pool of releasable glutamate in nerve endings. It has been demonstrated by DeBelleroche and Bradford (1977) that the source of glutamate released by depolarizing agents is a rapidly depleted cytoplasmic pool and not a stored vesicular pool, as is commonly thought to be the case for acetylcholine. It has been suggested by these authors, and by Hamberger et al (1979), that because turnover from other pools is insufficient to maintain levels of releasable glutamate during sustained activity, the majority of glutamate released is synthesised denovo. Neuronal activity is accompanied by an increase in metabolic activity in the nerve ending leading to an increased demand for glucose for central pathways and consequently a decrease in its ability to replenish cytoplasmic pools of releasable glutamate (Watkins, 1973). The neuronal pool of glutamate is characterized by the presence of large amounts of the enzyme glutaminase (Bradford and Ward, 1978), and it has been demonstrated by isotopic labelling studies that glutamine may be a much more efficient and specific precursor for releasable glutamate than glucose (Cotman and Hamberger, 1978; Bradford et al 1978; Hamberger et al, 1979). The activity of glutaminase would maintain high glutamate levels both by its direct

catalytic activity and by increasing the contribution of glucose to glutamate production as increased NH_4 both stimulates the activity of the glycolytic pathway and allows for an increased synthesis of glutamate via transamination with α KG (ketoglutarate). Rapid utilization of NH_4 in this way, and rapid release of newly synthesized glutamate, would further stimulate glutaminase activity by removing end-product inhibition. These studies provide convincing support for the view previously discussed that the glutamatergic neuron may depend for its activity on a closely woven interactive cycle of events involving glia and nerve endings. Glial cells, more particularly astrocytes, are rich in glutamine synthetase and could act as a store of glutamine produced from glutamate taken up from the synapse by the high affinity transport mechanisms. Using labelled glutamine as a precursor, Hamberger et al, (1978) have shown Ca^{++} dependent specific release of glutamate from slices of the dentate gyrus of the hippocampus but unfortunately there are no reports in the literature of the use of the same labelling paradigm in the measurements of glutamate release from cortical slices. The compartmentation studies on the role of glutamine are convincing enough to suggest that this would be a worthwhile approach for future release studies.

Other workers have avoided the use of exogenously applied isotopic precursors and have measured the total amino acid levels released from tissue slices following "physiological" stimulation using sensitive analytical techniques to discriminate between pre- and post-stimulation extracellular levels. Thus Ca^{++} dependent specific release of glutamate alone has been shown to occur from slices of olfactory cortex (Bradford and Richards, 1976) and of glutamate and aspartate from visual cortical slices (Clark and Collins, 1976). Yamamoto & Matsui (1976) have also demonstrated release of glutamate from olfactory cortex despite their use

of exogenously applied labelled glutamate, strengthening the candidacy of glutamate as excitatory transmitter utilized by the main excitatory input into the cortex. The same workers also highlighted another problem with amino acid release studies in that they were unable to demonstrate this release in a previous study (Matsui and Yamamato, 1975) and only succeeded by utilizing a reported blocker of high affinity glutamate uptake sites, L-cysteate, which they assumed were responsible in their first report for the immediate re-uptake of released glutamate leading to static levels of extracellular amino acid. Differences in methodology and sensitivity of analytical techniques utilized by the two groups of workers could account for this finding, but nevertheless the presence of such high affinity glutamate uptake systems must be considered in release studies utilizing heterogenous tissue samples.

The problem of heterogeneity, inherent in the use of tissue slices, has led to the widespread use of synaptosome preparations consisting of morphologically intact and metabolically active 'pinched' off presynaptic nerve endings, usually with adherent postsynaptic membrane. Thus any specific release of amino acids from these preparations must represent release from compartments functional in synaptic transmission. Another advantage of synaptosomal preps in this context is that release can be triggered in the same way by electrical pulses, high potassium concentrations or depolarizing agents such as veratrine. The efficacy of different physiological stimuli in evoking release in tissue slices varies considerably depending on factors such as the thickness of the slice and levels of electrical stimulation (Matsui and Yamamoto, 1975), or the accessibility of synaptic locations to K^+ concentrations (Johnson, 1979; Fagg and Lane, 1979). Glial cells in tissue slices are very potent in removing K^+ from extra-cellular medium (Henn, 1976, 1973) and may therefore protect neuronal membranes from this depolarizing influence.

The disadvantage of synaptosome preparations in the study of release criteria however, as pointed out by Bradford (1979), is the necessary loss of other structures such as the postsynaptic cell and glial cells that may be vital modulatory influences on the transmission process. This is especially important in view of the possible interactive cycle between glia and neurons at the glutamatergic synapse outlined above. Although synaptosomal preparations are not pure, with the degree of contamination by glial elements varying up to 50% (Henn and Hamberger 1973), the functional relationship between glia and nerve endings may be disrupted. Nevertheless, specific Ca^{++} dependent release of glutamate and aspartate from cerebral cortical synaptosomes has been shown (DeBelleroche and Bradford, 1972) and it is particularly interesting that this release was enhanced when the synaptosomes were preloaded with glutamate under high affinity conditions (i.e. in the presence of Na^{+} and concentrations of glutamate of 10^{-5}M - 10^{-6}M) that have already been shown to label a unique population of possible glutamatergic synapses.

Thus although considerable methodological problems are associated with the fulfillment of the release criterion leading to contradictory reports of the specificity of the release process for the putative transmitter amino acids as opposed to others (see Fagg and Lane (1978) for review), the accumulated data are on the whole an encouraging indicator of a synaptic role, at least for glutamate in the cortex.

In view of the difficulty in interpreting many of the metabolic studies of the role of glutamate, complementary techniques for the localization and visualization of possible glutamatergic nerve endings have proved extremely valuable. These techniques exploit the fact that amino acids can be transported into nervous tissue by transport mechanisms specific for different amino acid groups. All cells in the body, including those of the brain, possess these trans-membrane carrier mechanisms to ensure adequate supplies

of all amino acids for protein biosynthesis and central metabolism. These carriers have relatively low affinity for their substrates with affinity constants in the region of 10^{-3}M (1mM) (Blasberg, 1968; Blasberg and Lajtha, 1965). The search for mechanisms by which the transmitter amino acids could have their synaptic activity terminated led to the discovery that for these amino acids only, a high affinity transport with a K_M for the substrate of 10^{-5}M also exists (Snyder et al, 1973; Logan and Snyder, 1971, 1972; Balcar and Johnston, 1972; Benjamin and Quastel, 1976; Bennett et al, 1972; Divac et al, 1977).

As the amino acids also subserve a general function it seems unlikely that a degradatory enzyme analogous to AChE would be used to terminate their synaptic activity as the presence of such an enzyme may also interfere with supplies of the amino acids for other metabolic functions. The high affinity transport systems are also present in low concentrations and would therefore be sensitive to levels of amino acid transmitter likely to concentrate in the synaptic cleft, effectively removing the transmitter from the vicinity of the postsynaptic cell on cessation of the nerve impulse. There is a wealth of data supporting such a proposition. All other transmitters, with the exception of ACh, seem to be removed from the synaptic cleft in such a way. Thus high affinity transport systems have been demonstrated for serotonin (Kuhar et al, 1971), noradrenaline (Iversen, 1967), dopamine (Coyle and Snyder, 1969) and the established inhibitory amino acid transmitter GABA (Iversen and Neal, 1968). GABA is not an amino acid generally incorporated into proteins, it's only metabolic role being in synaptic function and, consequently, there is no low affinity transport system for GABA in the CNS, strengthening the case for the involvement of the high affinity processes in termination of transmitter function. Neurophysiological evidence shows that the amino acid glycine is a major

inhibitory neurotransmitter in the spinal cord although it is non-effective in hyperpolarizing cortical cells. In accordance with this is the finding that glycine is taken up into neural tissue from spinal cord and brain stem by both a high affinity and a low affinity system, whereas only a low affinity uptake system could be demonstrated in the cortex (Bennett et al, 1974; Werman & Aprison 1968, Logan and Snyder, 1971).

The density of high affinity uptake sites for the excitatory amino acids has been directly related to the increasing intracellular glutamate concentration from lower to higher brain areas (Johnson, 1978). This suggests the possibility that the function of the uptake sites would be to regulate extracellular levels of glutamate and aspartate maintaining synaptic concentrations below those necessary for depolarization. This may be essential in the face of a greater potential for leakage of the amino acid from neuronal tissue where a high intracellular/extracellular concentration gradient exists. In keeping with this is the well documented evidence that glia cells represent a major compartment for net accumulation of the amino acids by the high affinity process (Henn et al, 1974; Henn, 1976; Balcar et al, 1977; McLennan, 1976). This does not necessarily preclude a synaptic function for the amino acids, as careful modulation of extracellular levels are essential to maintain sensitivity of postsynaptic membranes to transmitter released by depolarization of the presynaptic nerve. On the other hand, glial uptake may also be essential were the amino acids not physiological transmitters, in order to prevent excitotoxic effects.

Much more indicative of a transmitter role for the amino acids is the wealth of data showing that potent high affinity uptake mechanisms also occur into synaptosomes (Bennett et al, 1972, 1973; Logan and Snyder, 1972; Roskoski, 1978; Iversen and Storm Mathisen, 1976). Moreover, synaptosomes prepared from the cerebral cortex are not uniform in their

capacity to accumulate the amino acids but can be separated by incomplete equilibrium sedimentation on sucrose density gradients into distinct subpopulations. Glutamate and Aspartate accumulated under high affinity concentrations can be localized to synaptosomes sedimenting in a less dense part of the gradient than those accumulating other amino acids and other neurotransmitter species, including GABA (Kuhar and Snyder, 1970; Wofsey et al, 1971; Bennett et al, 1972). That this phenomenon is not due to an uneven distribution of low affinity sites has been demonstrated by incubating synaptosomes with the amino acids in the presence and absence of sodium. In keeping with the ionic requirements of the transport systems for GABA and the catecholamines, the Na^+ dependency of the high affinity uptake of glutamate and aspartate, in contrast to the low affinity system which is not sensitive to Na^+, has been convincingly demonstrated in many types of neuronal tissue and would seem to be a general rule (Balcar and Johnson, 1972; Bennett et al, 1972, 1973; Peterson and Raghupathy, 1972; Wheeler, 1976). Thus, in the absence of sodium, all amino acids including glutamate and aspartate are evenly distributed throughout the whole sucrose gradient, whereas in its presence the unique profile is apparent (Snyder et al, 1973). The accumulated data is consistent with the presence of a proportion of glutamatergic nerve endings projecting into the cortex and differring in physical properties from other nerve endings releasing different transmitter species.

The uptake studies summarised here have been extensively criticised on several points that warrant careful consideration (DeFeudis, 1975). High affinity transport systems have been found in non-nervous tissue such as kidney and yeast (Neal and White, 1978) and may therefore subserve a common biological function unrelated to synaptic activity. However, it may also be the case that evolutionary advancement has lead to the efficient utilization of one system for more than one purpose, although

that begs the question of how the two functions of such a system may be differentially controlled. It may be that in some studies apparent uptake of radiolabelled substrate may not represent a net accumulation but a homoexchange with an endogenous pool of the substrate so that the specific activity in the extracellular medium falls without any alteration in concentration of substrate. Benjamin and Quastel (1976) have suggested that although homo- exchange does occur it does so by a different process of continually balanced influx and efflux of substances across the cell membrane which serves a homeostatic function. Unlike high affinity transport, homoexchange is not an energy dependent process and therefore cannot be prevented by inhibitors of transport process such as ouabain. Nevertheless, the contribution of homoexchange to changes in concentrations of exogenously applied putative transmitters remains difficult to estimate and control for and may therefore leave room for errors in the interpretation of uptake data. It may also be important to consider that while high affinity uptake is sensitive to Na^+ ion concentration in vitro, such sensitivity may not be significant in vivo where Na^+ concentrations are unlikely to change to the extent where it could be a useful control mechanism for the process.

So while the demonstration of a high affinity transport process for the putative amino acid transmitters in some nerve endings points towards a function in synaptic transmission, and a lack of such a system in any one area would be difficult to reconcile with transmitter activity, other interpretations of the data may be possible. A more coherent approach may be to systematically study regional differences in high affinity processes, linked to the functional activity of different projection pathways. Following this line of investigation it has been shown that axotomy of the corticostriatal pathway leads to a significant decrease in high affinity uptake of glutamate into neostriatal synaptosomes (McGeer et al, 1977) and

into hippocampal synaptosomes after lesioning of the commisural path (Storm-Mathisen, 1977). That such differences may be a solid neurochemical marker for putative transmitter systems is demonstrated by the fact that many axotomies in higher C.N.S. regions did not effect glutamate uptake in the projection sites (Margolis et al, 1974; McGeer et al, 1977; Storm Mathisen, 1977). Similar studies of possible projection pathways into the cortex may show variations in glutamate or aspartate high affinity uptake that may correlate with differences in other neurochemical parameters such as intracellular concentrations, levels of specific release or densities of post-synaptic receptor sites.

Although glutamate and aspartate are extremely effective in eliciting depolarization of cortical neurons upon electrophoretic application (Curtis and Watkins, 1963), there are small differences between electrically stimulated and K^+ evoked changes in membrane potential and changes evoked by applied glutamate. In particular, the reversal potential for glutamate is significantly different to that of the natural transmitter (Zieglgansberger, 1973; Curtis, 1965). This may be related to the observation that excitatory synaptic inputs are differentially located being mainly dendritic, whilst iontophoretically applied glutamate will excite via perikaryal receptors predominately, so that observed membrane depolarizations may be a summation of the effects of receptors with different physiological functions, one of which may be unrelated to synaptic transmission. An electrophysiological analysis of the role of glutamate is complicated not only due to the uncertainity of the exact location at which the amino acid is acting and its function at each site, but also due to the probable ability of glutamate and aspartate to cross react with each others receptors. Both are flexible molecules and such cross reactivity may be due to the ability of their carbon chains to fold or extend whilst maintaining their ionizable amino and acidic groups

in the correct alignment for interaction with their opposing binding sites on their receptors. If glutamate interacts with its own receptor in an extended conformation then the ability of aspartate to bind to these glutamate preferring receptors may be limited. So the problem of satisfying the postsynaptic criteria for the amino acids becomes one of differentiating between sites of interaction of the amino acids with nerve cell membranes. If they are to serve a physiological function in synaptic transmission, postsynaptic receptors for neurotransmitters should exist in low finite numbers and have a high affinity for their natural ligand and thus should saturate at relatively low synaptic concentrations of transmitter (Burt 1978).

Kinetic characterisation of binding sites may therefore allow discrimination between such receptor sites and those which subserve a different function, e.g. transport sites for the accumulation of the amino acids for metabolic purposes. Binding sites for neurotransmitters should also exist in a particular conformation so that specificity of interaction is ensured - post synaptic events triggered by interactions between transmitter and receptor must not be accidently initiated by other ligands. Thus the structural requirements for effective interaction should be unique for each receptor type. Binding studies utilizing putative transmitter agonists and antagonists radio-labelled to a very high specific activity is now a well established technique allowing the direct study of such interactions (Snyder, 1975; Snyder and Bennett, 1976).

Several workers have investigated <u>in vitro</u> the binding of tritiated L-glutamate to membranes prepared from cerebral cortical tissue (DeRobertis and DePlazas, 1976; Lahdesmaki and al, 1977; Michaelis et al, 1974; Roberts 1974) and other brain areas such as cerebellum (Foster and Roberts, 1978; Sharif and Roberts, 1981), hippocampus (Baudry and Lynch, 1979, 1981) and

striatum (Vincent and McGeer, 1980). Although such studies have demonstrated characteristics of the binding that are at least consistent with a neurotransmitter role for glutamate, there is considerable variation between the methods used and the findings of the different groups of workers. These results are summarized in Table 51, later in the thesis.

Consideration of the accumulated data reported here in terms of fulfillment of the classic neurotransmitter criteria strongly favour a synaptic role for glutamate whilst nevertheless highlighting problems of experimental design, methods of interpretation and depth of knowledge relating to the different approaches.

Despite variable results between different binding assays, the increasing emphasis on the application of such methods is a reflection of the fundamental need for a definition of the observed excitatory activity of the acidic amino acids in terms of the molecular events specifically initiated by the binding of each putative transmitter species.

CHAPTER 2

EXPERIMENTAL APPROACH

2.1 ONTOGENY

Although the criteria provide guidelines for investigating the candidature of putative neurotransmitters it remains essential to demonstrate that not only do the requisite individual biochemical parameters exist, but that they represent component parts of an integrated neurotransmitter system. One valuable approach to this problem is to monitor the concerted development of different neurochemical correlates of transmission. The rationale being that during brain maturation when complex neuronal circuits are being laid down, a better understanding of the functional importance of the processes thought to mediate neurotransmission may be obtained by examining ontogenetic relationships between the components of a system, as opposed to studying a single component in isolation.

An ontogenetic approach to the problem of transmission mediated by acidic amino acid candidates can be justified on further grounds. In general terms, any information concerning the stage of development at which a particular neuronal pathway appears could have important implications for the assessment of the influences of that pathway on subsequent commitment of neurons in functionally or morphologically related areas. Neuronal activity does appear spontaneously prior to the development of presynaptic input, but at least with catecholaminergic neurons it has been shown that 95% of the functional commitment of the neurons to that particular mode of neurotransmission, by differentiation, occurs only after innervation (Coyle 1976). It is therefore highly probable that the development of receptors on the soma of neurons does have a role in modulating their differentiation. Any knowledge of factors controlling such differentiation could have important clinical application in monitoring foetal brain development.

A difference in the developmental profile of the processes that mediate neurotransmission could also reflect the different functions that neuronal circuits perform at various stages during ontogeny. Indeed, the pathways that have been rigorously studied ontogenetically, mainly catecholaminergic (Coyle, 1974; Olsen et al, 1973; Coyle and Campochiaro, 1976; Lamprecht and Coyle, 1972), cholinergic (Coyle and Yammamura 1976; Yammamura and Snyder 1974; Simon et al, 1976; Hattori and McGeer, 1973) and striatal gabaergic (Coyle and Enna, 1976), do exhibit major differences in the time of their appearance and their rate of differentiation.

The cerebral cortex of the rat provides a good model for the study of the biochemical correlates of developing transmitter systems because of its relative immaturity at birth, Although the neonate possesses the full complement of presumptive cortical nerve cells their differentiation is incomplete. These neuroblasts are tightly packed together at this stage

and become separated during the course of the first postnatal week by the outgrowth of neuronal processes between the cell bodies. The greatest contribution to the doubling in cortical thickness during this stage is the proliferation of the dendritic tree, although axonal growth is also taking place (Caley, 1971). Simultaneously, there is a dramatic increase in soma size characterised by the development of a complex and extensive rough endoplasmic reticulum and accompanied by a very high rate of protein synthesis. This is reflected in the appearance of specific cell surface proteins and glycoproteins involved in a multiplicity of roles (e.g. as enzymes, structural proteins and in cell adhesion and recognition phenomena) but which undoubtedly include the functioning and modulation of transmitter systems. Thus the neurochemical specification of synapses is essentially a postnatal event. Neither are the definitive physical features of mature synapses present prior to 10 days of age, although presumptive synapses are easily identifiable. By 12 days of age many mature synapses can be recognized but maximum rates of synaptogenesis do not occur until around 20 days (Aghajanian and Bloom, 1967).

In pursuit of an interest in both the mechanisms by which neuronal pathways become specified and the vexed question of whether or not the acidic amino acids fulfill a neurotransmitter role in the cortex, this thesis reports the utilization of binding assays to characterize both the adult features and the ontogeny of pre- and post synaptic neurochemical markers that may serve to mediate glutamatergic and aspartergic transmission.

2.2 Receptor Theory

The investigation of the interaction of glutamate and aspartate with synaptic membranes utilizing binding assay techniques offers many advantages

as an approach to clarifying their neurotransmitter status. In particular, binding assays are based on relatively simple and easily accessible technological principles. Innovations in methodology in recent years are mainly the result of a firmer theoretical basis concerning the probable mechanisms of interaction evolved from earlier studies and are relatively independent of the constraints, both practical and financial, posed by a reliance on increasingly sophisticated technological developments inherent in some other approaches. In addition, the availability of radioligands labelled to an extremely high specific activity has allowed the detection of very low levels of interaction.

Ligand binding studies are invaluable not only in quantifying levels of interaction with nerve cell membranes, but also in distinguishing between and characterizing different types of recognition site on a qualitative basis. This can be achieved by many different manipulations. For example, the inhibition of any binding of radioligand in the presence of analogues may be studied so that the structural requirements of the binding sites may be defined. The medium in which the assay is performed can be altered to study the influence of various ions on the binding process, and in a similar way the influence of factors such as pH and temperature, over physiological ranges, may be examined. In addition, the influence on the binding of various agents both chemical and enzymic with known specific effects on membrane components can be studied to gain information on the macromolecular identity of the recognition site.

Such a detailed quantitative and qualitative analysis of recognition sites for the putative transmitters on nerve cell membranes may allow a much more decisive discrimination between possible physiological roles. Such discrimination becomes vital when transmitter candidates such

as the excitatory amino acids are considered as they also serve a major function in intermediary metabolism apart from any synaptic role and will therefore bind to transport sites subserving this metabolic function.

In a wider context and as pointed out by Ariens and De Miranda (1979), the development of binding assays could have important implications for the understanding and treatment of disease states, allowing as they do for a comparison of the binding capacity of receptor systems in normal and pathological conditions and the efficacy of binding of various drugs under these different conditions.

Undoubtedly, the most important proviso in the utilization of a binding assay and the interpretation of ligand binding data is the recognition that what is being studied is simply the initial link in an inexorable chain of events that leads to the manifestation of a biological response, which in the case of neurotransmitter action is almost certainly the opening of membrane ion channels. Binding assays must therefore be carried out only within a paradigm suggested by an understanding of the receptor concept which attempts to explain the initial recognition and binding event in terms of its translation into an intracellular response. Information concerning ligand/receptor interactions gleaned from <u>in vitro</u> binding studies can only be significant if the data is at least compatible with an ability to initiate such cellular responsiveness.

As previously mentioned, on the basis of levels of transmitter agonist needed to evoke a measurable electrophysiological response in the transmitter systems so far studied, it is reasonable to assume that postsynaptic receptors for putative transmitters will be present in very low concentrations so that they will quickly become saturated with an increase in concentration of transmitter and that the affinity for the reaction will be high. In addition such interaction must be reversible to account for the observable cessation of response on removal of the stimulus.

These two kinetic parameters, maximum density of binding sites and affinity constant, cannot be easily estimated from an analysis of dose-response curves, however, without making several assumptions as to the nature of the coupling mechanism between agonist binding and response.

The relationship of concentration of ligand used to elicit a response is evaluated within the mathematical framework designed to assess substrate-velocity relationships for enzymic reactions. This interpretation assumes the magnitude of the biological response to be proportional to the number of receptors activated by complexing with the ligand, so that the maximum response, Qmax, is achieved when all the receptors are occupied with ligand. This is based on a fundamental tenet, first proposed by Ehrlich in the first decade of this century, that for an agent to act it must be bound. In keeping with the law of parsimony the process of receptor/ligand complex formation can then be seen as a simple bimolecular interaction where

$$R + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RL \longrightarrow \text{Response}$$

Where L is equal to transmitter agonist, R = receptor and RL is receptor ligand complex. At equilibrium k1 = k-1, so that the equilibrium binding constant may be expressed either as an association constant KA or, as used

throughout this thesis, the dissociation constant KD. Then :-

$$KD = \frac{k-1}{k1} = \frac{(R)\ (L)}{(RL)}$$

Thus the biological responsiveness of a system, Q, is related to the affinity constant of the receptor for its agonist by the following equation:-

$$Q = \frac{Qmax \times (L)}{KD + (L)}$$

which is exactly analogous to the classic Michaelis-Menten equation for analysis of substrate-enzyme interaction. The shape of the dose response curve predicted by the equation is thus hyperbolic and the concentration of agonist giving a half maximal response (ED5o) is equal to the affinity constant of the receptor for its ligand. (L) in this case is the total concentration of ligand present in the system which is assumed to be equal to the free concentration of ligand on the basis that, given a very low receptor population, the proportion of bound ligand, and thus receptor ligand complex (RL), will be negligably small. However, as previously mentioned, the accurate determination of synaptic concentrations of iontophoretically applied ligand is extremely difficult. Contributory factors to this problem are limited knowledge of the volume of intercellular space into which the agonist is injected, the location of the active synapse in relation to the site of application, the extent of diffusion away from this site and the activity of mechanisms designed to remove neuroactive substances from the synaptic cleft. So, a major initial assumption is that the concentrations of transmitter or agonist reaching the postsynaptic receptors is not significantly different from the initial concentration of ligand apllied, specified in terms of the current used for its ejection from the micropipette. In addition, although this occupancy theory of the mechanism

of agonist action has been extensively developed by many workers (Ariens and Simonis, 1964; Stephenson, 1956; Stephenson and Barlow, 1970), there are several experimental observations that cannot be adequately explained by such theories and which render the assessment of kinetic parameters difficult, if not impossible, by the direct study of the responsiveness of a biological system.

In particular, studies examining the effect of irreversible antagonists on the response elicited by both hormone (Seelig and Sayers, 1973) and neurotransmitters (Van Rossum, 1972) have shown that low concentrations of such antagonists do not affect the maximum response elicited by the agonist, although this response is reduced at higher concentrations of antagonist. This has been interpreted as due to the presence of spare receptors or a receptor reserve so that only a certain fraction of the total receptor population need be occupied for maximal effect. This situation can be rationalised in terms of the effectiveness of systems designed to amplify the initial signal of receptor/ligand binding. A single ion channel may be opened by any one of a number of activated receptors allowing for large changes in transmembrane potential. Thus an irreversible blockade of a proportion of those receptors could still be tolerated if others remained free to bind agonist and cause channel opening.

Partial agonists are substances capable of binding to a receptor, but which are much less efficient in forming an activated complex with the receptor (Ariens, 1954, Ariens & Simonis 1964). For these substances maximal response can only be attained at full receptor saturation. In confirmation of the existence of spare receptors the maximal biological responsiveness elicited by partial agonists is immediately reduced in the presence of the same low levels of antagonist that fail to decrease the response to a full agonist.

Many such studies suggest that spare receptor capacity may be a general rule (Ariens & De Miranda, 1979; Cuatrecasas and Hollenburg, 1976). Accordingly the second authors have estimated that in all cases where more receptors are present than the number required for a maximal response, the concentration of agonist required for ED_{50} (concentration eliciting half maximal response) will underestimate the true value of the KD.

The situation may also arise, that a minimal proportion of a receptor population may need to be occupied before any response at all is detectable and that proportionality of response to receptor occupancy only occurs after this threshold has been reached (Cuatrecasas and Hollenburg, 1976; Hollenburg, 1978). In such a situation it is possible to deduce that the ED_{50} will always overestimate the value of the true KD.

In vitro ligand binding studies obviate these problems (although admittedly introducing others!) by using cell free membrane preparations so that the initial recognition event can be analysed quantitatively without the need for assumptions as to the mechanism of coupling. However, although agonist affinities cannot be estimated in vivo, the same is not true for antagonist affinities as in this case it is the inhibition of responsiveness, rather than responsiveness per se, that is being measured. Thus antagonist equilibrium constants can be measured accurately by measuring the concentration dependence of that antagonists' ability to prevent access of agonist to the receptor. The mathematical model used to derive the equilibrium constants (Schild, 1949; Arunlakshana and Schild, 1959) rest on the principles of the null hypothesis, whereby it is assumed that, when the response to one concentration of agonist in the absence of antagonist is the same as the response to a higher concentration of agonist in the presence of antagonist, then the amount of agonist reaching the receptor in the two situations must be identical. The point

here is that antagonist affinities measured in this way provide a parameter that should have the same value whether derived from *in vivo* or *in vitro* studies if the same recognition site is being studied, and thus may provide the means of assessing what exactly is being looked at in ligand binding studies!

Also, *in vivo* experiments analyzing dose effectiveness of ligands, although being of very limited use in providing quantitative data concerning the nature of the ligand/receptor interaction, can be used very effectively in studying the relative potencies of a series of analogues to elicit a similar response. *In vitro* binding studies utilizing the same series of analogues should show increasing affinity paralleling increasing responsiveness if the same receptor is being measured. Thus once more, *in vivo* studies of responsiveness provide an important context for assessing the physiological relevance of the binding estimated *in vitro*.

In view of the inadequacies of the occupancy theory to provide a framework for the understanding of the biochemical mechanism by which transmitters exert their effect, other theories have been developed in an attempt to provide both an explanatory paradigm for interpretation of physiological and ligand binding data and a conceptual framework, upon which ligand binding experiments may be constructed.

Most recent developments in receptor theory, not only attempt to analyse the dynamics of receptor/ligand interactions, but also try to account for the subsequent event in the evocation of the physiological response, namely the relationship between the activated receptor and an effector molecule.

The observation of sigmoid dose response curves in particular, led to the development of cooperative models for ligand interaction based on their similarity to the sigmoid velocity/substrate relationship exhibited by allosteric enzymes. Thus an initial development was the adaptation of the Monod-Wyman-Changeux model for the nature of allosteric transitions to account for the complexity of such dose/response curves. The receptor is visualized as a membrane localized complex with the ionophore. This complex or drug-binding protomer can exist in one of two states, each with greatly different affinities for agonists and antagonists. In the absence of any ligand, the protomer will adopt an equilibrium between the two states, T (closed) and R (open). The affinity of agonists is proposed to be much greater for the R state than for the T state. Thus agonist binding will shift the equilibrium to the R state leading to channel opening. Antagonists will exert an opposite effect. Such a model also accounts for densensitization phenomena where the recognition molecule has become uncoupled from the ionophore. The dissociated receptor may then exhibit ligand binding affinities different from either the associated R or T state which would undoubtedly be reflected in the observation of sites with different affinities in in vitro ligand binding studies.

Cooperative effects have been incorporated into this model by Changeux et al (1970) who proposed that the concerted transition of a number of linked protomers to the R state may be necessary before channel opening is achieved, the initial binding of ligand to one protomer facilitating subsequent binding to other protomers. This is exactly analogous to the cooperativity of subunits within the haemaglobin quaternary complex upon binding oxygen. Karlin (1967) alternatively proposed that a certain number of independent protomers may need to be in the R state simultaneously before channel opening and thus presented an

explanation for the threshold effect not accommodated with the occupancy model. Perhaps the crucial feature of both models is that they provide a theoretical framework for evaluating binding data that not only exhibit receptor populations that will either bind agonist or antagonist with high affinity but also with differing affinities (high and low) for any particular ligand. Partial agonists in this context would have some affinity for both R and T states. These observations imply that during the fractionation procedure for isolating membranes from nerve tissue the equilibrium between the R and T forms will be frozen so that the receptors will behave as two distinct populations. In addition, isolation procedures performed at different times might certainly be expected to yield different proportions of the R and T form depending on the equilibrium position attained immediately prior to the beginning of the experiment. This may account for large variations in the levels of binding between experiments.

However, whilst accounting for many observed in vitro and in vivo effects the two-state models do impose certain restrictions in that they presuppose an association of the ligand recognition site with its effector.

An extremely elegant model for explaining the relationship between the recognition event and the physiological effect was simultaneously suggested by Cuatrecasas and Hollenburg (1976) and DeHaen (1976). Both authors proposed that the mobility of receptor proteins within their lipoprotein environment would be strongly influenced by the binding of antagonists and agonists with differing affinities for hydrophobic or hydrophilic binding sites on the receptor molecule. This mobile receptor hypothesis has been extensively reviewed by many workers (see e.g. Ariens and Miranda 1979). In essence it proposes that the binding of polar agonists could

alter the interface between receptor molecule and lipid membrane components exposing hydrophilic portions that would therefore exhibit a tendency to aggregate either among themselves or with other hydrophilic membrane components, e.g. ionophores. The binding of non-polar antagonists would similarly strengthen and fix the position of the receptor within the membrane by favouring the maintenance of hydrophobic interactions with the lipid environment, thus preventing lateral mobility and effector activation. The elegance lies in exploiting the current concept of the fluid mosaic nature of biological membranes as well as introducing considerable flexibility into the possible mechanism of action of transmitters. As explained by Hollenburg (1978) such models can account for many observed in vivo effects (e.g. denervation supersensitivity, evoked by changes in receptor/effector ratios) and in vitro binding effects including different binding affinities (non-linear Scatchard plots), negative and positive cooperativity (Hill plots >1 to <1) and spare, but potentially equivalent, receptors.

The transduction mechanism by which transmitter/receptor interaction is converted to changes in ion gating may however be more complex than the mobility theory allows. This conjecture is based on strong evidence that receptors for many hormones and neuro-humoral factors (prostaglandins, opiates and angiotensin) are linked within the membrane as an oligomeric complex with GTP regulatory subunits (N). Binding of hormone to this R.N. complex leads to the formation of H.R.N. monomers and has the primary effect of increasing the ability of N to bind GTP. Only the H.R.N. GTP monomer complex is capable of activating (or inhibiting, depending on the nature of N) the effector, (C), be it enzyme or ion gate. This model, reviewed and outlined by Rodbell (1980), allows for the alteration in the activity of a system under different physiological conditions, by changes in R.N. subunit structure, by altering R.N./C ratio by effecting the

degree of coupling between R and N or by controlling the availability of GTP. If this reaction mechanism is generally applicable it would mean that two ligands (hormone or transmitter and GTP) would be essential for physiological action. The observed response would then only be an accurate reflection of binding affinity for the first messenger if GTP was present in unlimiting amounts.

The implications of this theory for the interpretation of binding studies carried out in vitro are manifold. Evidence from hormone binding studies using both isolated membranes and intact cells suggest that the availability of G.T.P. may modify the affinity of the receptor for its ligand. Thus different Kd estimates may arise in different experimental systems depending on levels of the nucleotide. Different agonist affinities have also been observed with free receptors as opposed to .R.N. complexes with free R having the lower affinity. If this were a general case then procedures for isolating membranes for use in in vitro assays may cause disruption of R.N. units to a greater or lesser degree leading also to the expression of different binding affinities in different studies. Free R units may be normally present in the membrane, being formed from dissociation of R.N. units and en route for endocytotic removal.

It is thus obvious that ligand binding studies, whilst potentially providing essential information concerning the nature of receptor ligand interactions, can only do so if they are planned and evaluated within the context of a theoretical knowledge of the kinds of receptor model that can account for the observed in vivo effect, and the implications of such models for generating complex binding data.

2.3 The Binding Assay

Although in vitro binding assays utilizing putative neurotransmitters labelled to a high specific activity may be the only way of characterising receptor sites and quantifying receptor/ligand interactions, it is essential that certain precautions derived from theoretical considerations are followed. If a measure of the Kd is to be truly representative of the affinity of a ligand for its receptor then it is essential that it should be measured when the system has reached equilibrium or steady state, when the values of (RL) and thus (R) and (L) maintain a constant value. This occurs when the rate of the forward reaction equals the rate of the reverse reaction in the bimolecular rate equation. It is therefore necessary to establish the physical and biochemical conditions which will allow equilibrium to be achieved, within a realistic timescale. This in itself may involve a conflict of interests between optimising the binding whilst maintaining the integrity of the reactants.

One of the most crucial criteria for the identification of any binding sites measured in the binding assay as transmitter receptor sites, is that the binding should be saturable. There should only be a small finite number of receptor sites per mg tissue. It is therefore, important to quantify this number, or Bmax, where Bmax = (RL) + (R). The maximal number of binding sites is related to the affinity constant by the following equation:-

$$(RL) = \frac{Bmax\ (L)}{(L) + kd}$$

which is again identical to the classic Michaelis-Menten plot for enzyme substrate interactions. A first approximation of the binding constant may be obtained by the generation of a ligand displacement curve, using a small concentration of radioligand in the binding assay and adding

increasing amounts of unlabelled ligand. The concentration of unlabelled ligand at which maximum binding is displaced by 50% (IC_{50}) should (allowing for certain assumptions) approximate to the kd value. A more accurate estimate is obtained from saturation studies in which the concentration of radioligand is progressively increased in the medium. Plotting the amount of radioligand bound per mg tissue at each concentration (B) against the free concentration (F) will result in a hyperbolic curve (assuming a bimolecular reaction). Bmax and the affinity constant can be best obtained by transforming the data to fit a linear equation:-

$$B/F = \frac{Bmax - B}{B}$$

which is the Scatchard equation. Plotting the ratio B/F against (B) should give a straight line, if binding is saturable, which crosses the x axis at Bmax and whose slope = Kd. The crucial factor is that it is essential to know the concentrations of ligand (bound and free) at equilibrium. The simple option is to assume that the actual free radioligand concentration is not significantly different from the concentration of added radioligand. However, this assumption is obviously only valid when a small proportion (less than 10%) of the radioligand is bound at equilibrium(Bennett, 1978) thus enough radioligand must be added to ensure this excess.

The most considerable problem though, would seem to be identifying that proportion of the total binding that <u>is</u> to physiologically active receptors and not to a vast number of non-specific sites. It is very well documented from the literature on insulin and opioid binding studies that everything sticks to everything. (Cuatrecasas and Hollenberg 1975, Snyder et al 1975). It is thus essential to account for this non-specific binding by adding to a 2nd set of tubes containing the radioligand a large excess of the same ligand in a non - radioactive form. If all the sites on the neuronal membranes are very low affinity binding

sites present in very large numbers then binding should carry on increasing in proportion to concentration, so that the amount of radio ligand bound should not be decreased by these high concentrations. However, if there is present a population of saturable high affinity or "specific" sites then the very high levels of non-labelled drug should ensure that the labelled ligand is competitively prevented from binding to these sites leading to a reduction in the total amount of bound radioactivity. Any radiochemical that is bound will be to low affinity unsaturable "non-specific sites". Subtracting this amount of bound radioligand from the amount bound in the absence of excess unlabelled ligand should lead to an accurate estimate of the specific saturable binding component.

The next problem is posed by the selection of a suitable technique for separating the bound from the free ligand for measurement. As soon as the equilibrium position is disrupted the bound ligand will begin to dissociate from its receptor and will be lost, leading to an underestimate of binding. So the major constraint on the choice of separation technique is the rate of dissociation of the receptor/ligand complex. From theoretical considerations, to avoid the loss of more than 10% of bound ligand, the separations must be complete in .15 of the time taken for the receptor ligand complex to dissociate by 50% ($t_{\frac{1}{2}}$) (Bennett, 1978). The dissociation half-life is quite clearly related to the affinity of a receptor for its ligand, higher affinities (lower Kd values) generally corresponding to slower dissociation rates (Burt, 1978). The rate of dissociation may of course also be decreased by lowering the temperature at which the separation takes place. Compromises are involved with either of the two main methods of separation commonly employed in binding studies, namely filtration or centrifugation. Nevertheless, for receptor binding studies exhibiting affinity constants of less than 10^{-8} M

centrifugation is the method of choice, as for much of the time period of separation the equilibrium existing at the end of the incubation period prevails. (Bennett, 1978). Another important consideration is that precautions must be taken to ensure that the radioligand utilized in the binding assay is not metabolised to any appreciable extent. If this were so then it may be the metabolite which is binding to the tissue being studied and not the added radioligand. This problem may be considerable in studies of putative transmitters that play a major role in metabolism, such as glutamate and aspartate. Methods of minimising this problem include the thorough washing of tissue samples to ensure that there are no adhering metabolizing enzymes and limiting any residual enzyme activity by carrying out the binding assay at low temperatures. This last precaution may also, of course, considerably affect the rate at which equilibrium between the ligand and the receptor is obtained, the decrease in kinetic energy at the lower temperatures considerably lengthening the necessary incubation time. This then is the theoretical and methodological background upon which the following binding studies were developed and carried out.

CHAPTER 3

MATERIALS AND METHODS OF TISSUE PREPARATION

Materials

The brain membranes used in these experiments were prepared from Wistar rats (Strain CFHB), 0-50 days of age. The cerebellar mutant mice used in the binding studies were raised on a C57 BL/6 background with controls coming from a mutant-free strain with the same genetic background. Below 15 days it is difficult to recognise the mutation on a behavioural basis, and it is only due to the technical expertise within the animal house at the O.U. that reelers have been maintained beyond this age. L-^{3}H-Glutamate , L-^{3}H-Aspartate and ^{3}H-kainic acid of varying specific activities (between 6-34Ci/m.mol) were obtained from the Radiochemical Central Amersham. Scintillation fluid, Cocktail T, was obtained from Hopkins and Williams. All other chemicals were obtained from Sigma Chemicals, U.K.

Tissue Preparation

Rats and mice were decapitated and the cerebral cortex was rapidly removed and placed on ice. White matter and underlying structures were carefully dissected off and the tissue was homogenized in 10-20 vols. of ice-cold 0.32M Sucrose using 6 passes in a glass homogenizer fitted with a Teflon pestle. The homogenate was then centrifuged for 10 minutes at 1,000g and the pellet (crude nuclear fraction) was discarded, to yield SN1.

Preparation of P2 Fraction

The homogenization of brain tissue in iso-osmotic sucrose solutions has been shown to be favourable for the formation of nerve ending particles (Whittaker and Gray 1962). Dendrites and cell bodies swell and disintegrate, at the same time, nerve endings shrink and pull away from their attachments. These nerve-ending particles are recovered during subcellular fractionation proceedures in the same fraction as mitochondria i.e. essentially a P2 fraction (Hebb and Whittaker 1958, Gray and Whittaker 1960, 1962).

The supernatant (SN1) was centrifuged at 12,000g for 20 mins to provide a P2 fraction according to classic cell fractionation procedures. (See Duve, C de 1959). The resultant pellet was resuspended in a hypotonic buffer (20 vols. 5mM Tris HCl pH 8.4) and left on ice for 30 min. to ensure lysis of any vesicles present and the release of endogenous L-Glutamate. The suspension was then centrifuged for 10 mins. at 48,000g. The pellet was washed twice by resuspending in 50mM potassium phosphate buffer pH 7.1 and recentrifuging. The pellet was finally resuspended in the same buffer for use in the binding assay.

Electron micrographs of P2 fractions prepared from rats at different age points are shown in Plates 1-4. Although all of the samples are

necessarily heterogenous, nerve-ending particles are apparent even in the fractions prepared from 6 day old rats (Plate 1). The micrographs show very little tissue in the form of free membrane fragments especially in the P2 fractions prepared from rats between 6 - 20 days of age. Mitochondria are present in all fractions though the integrity of the organelles is poorly preserved as a result of the hypo-osmotic preparation conditions. All of the fractions are relatively uncontaminated with myelin, as great care was taken to clean the cortices of white matter before the fractionation process was carried out.

Preparation of Crude Synaptic Membranes

This procedure is essentially as described by Simon et al (1976) and is as follows. SN1 was centrifuged for 20 minutes at 17,000g. The pellet was resuspended in ice-cold distilled water by hand homogenization. This suspension was then centrifuged for 20 minutes at 8,000g. The supernatent fluid along with the soft upper layer of the pellet was collected. This supernatant fraction was then centrifuged for 20 minutes at 48,000g. The pellet was washed twice by resuspending in ice-cold distilled water and recentrifuging, before final resuspension in 50mM potassium phosphate buffer for the use in the binding assay.

PLATES 1 - 4

Electron micrograph of P_2 fractions prepared from the cerebral cortices of rats at different age points.

Plate 1 - 6 day

Plate 2 - 10 day

Plate 3 - 20 day

Plate 4 - 50 day (Post-lysis)

Subcellular fractions were fixed in 3% glutaraldelyde in 100mM sodium cacodylate - HCl/100mM sucrose buffer (pH 7.4) for 12 hours at 4°C and post-fixed in 1.3% osmium tetroxide in 67nM s-collidine (2, 4, 6 - trimethyl pyridine) - HCl buffer (pH 7.4) for 2 hours at 4°C and then 1 hour at 20°C. After dehydration the tissue was infiltrated with epoxy resin, embedded, cured and ultrathin sections (60 -90nm) cut on a Reichert OM U3 microtome. Sections picked up on 200 mesh uncoated copper grids were stained in a saturated solution of uranyl acetate in 35% methanol followed by lead citrate. Sections were viewed and photographed in a Phillips 301 transmission electron microscope.

Plate 1 - 6day

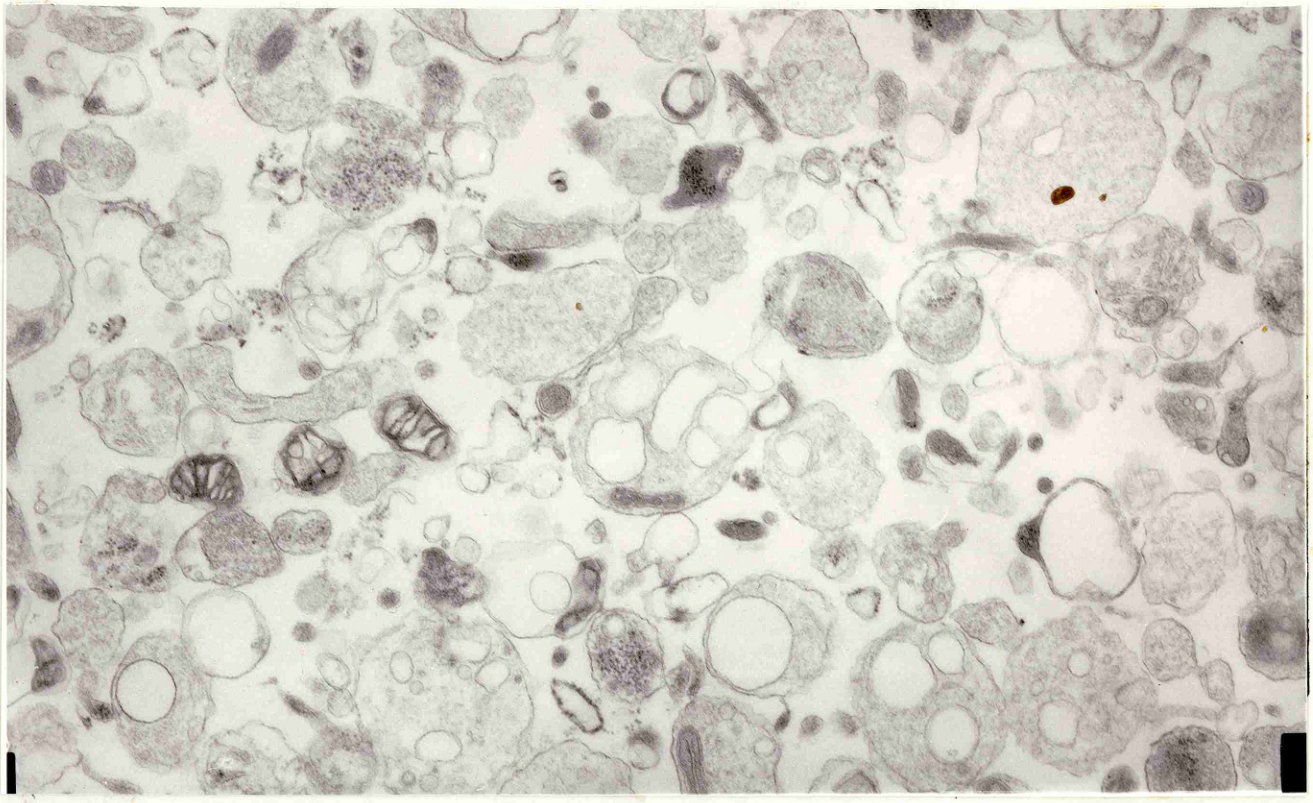


Plate 2 - 10day

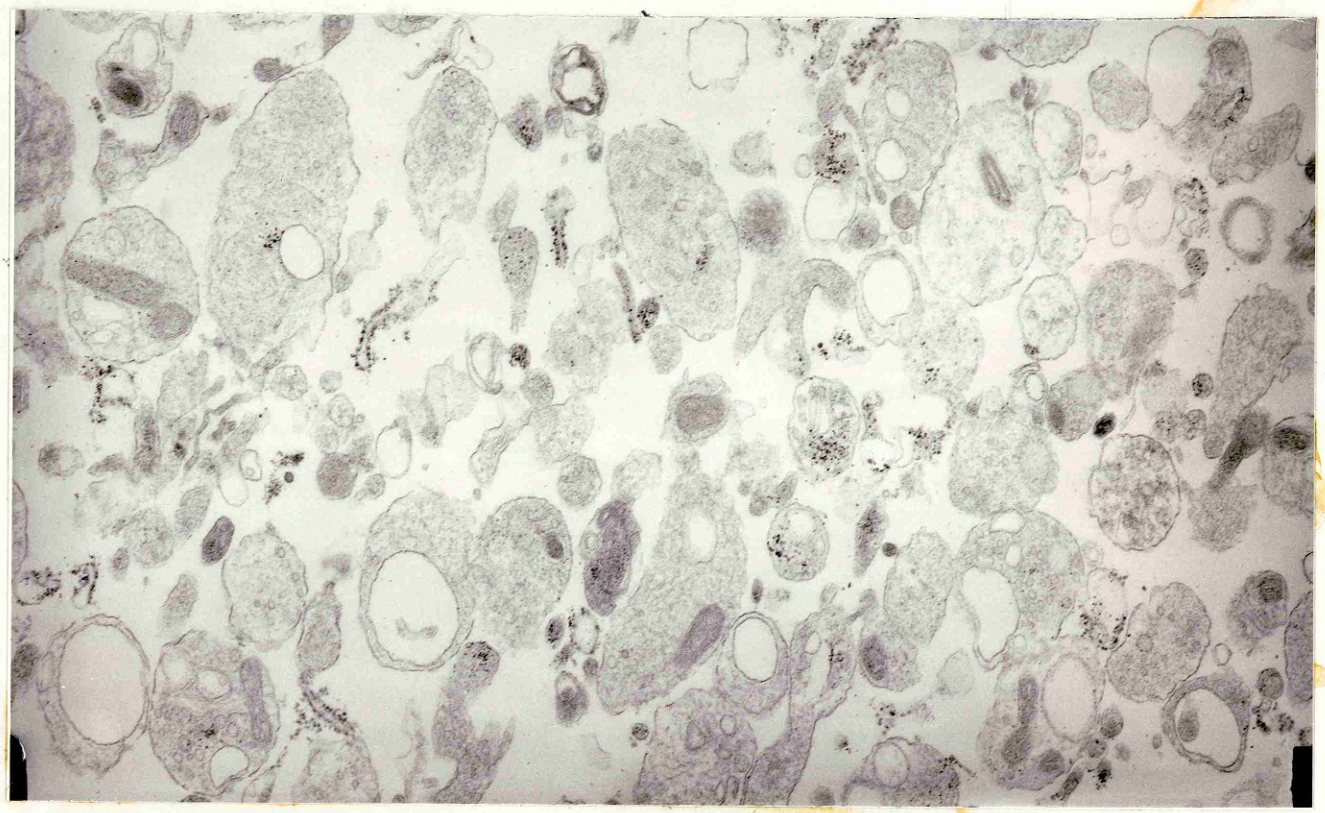


Plate 3 - 20dy

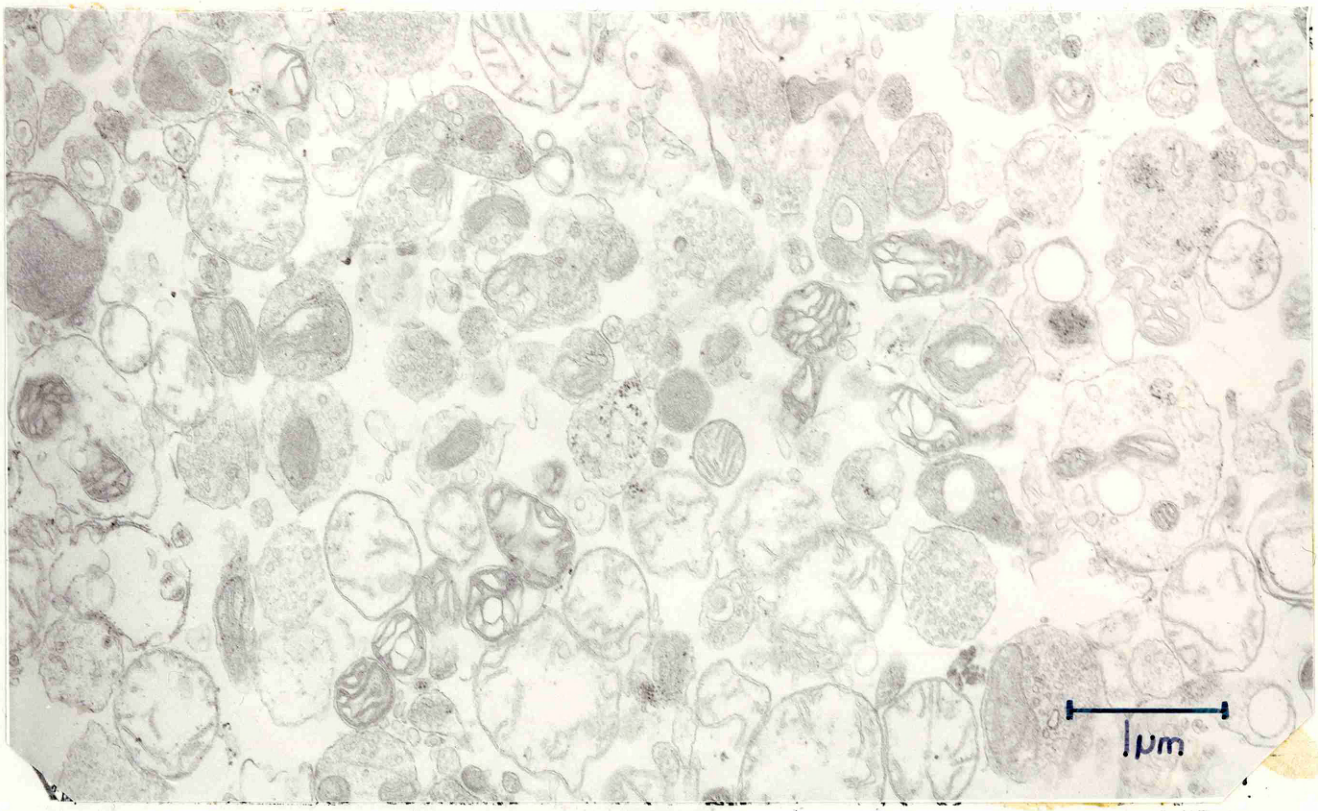
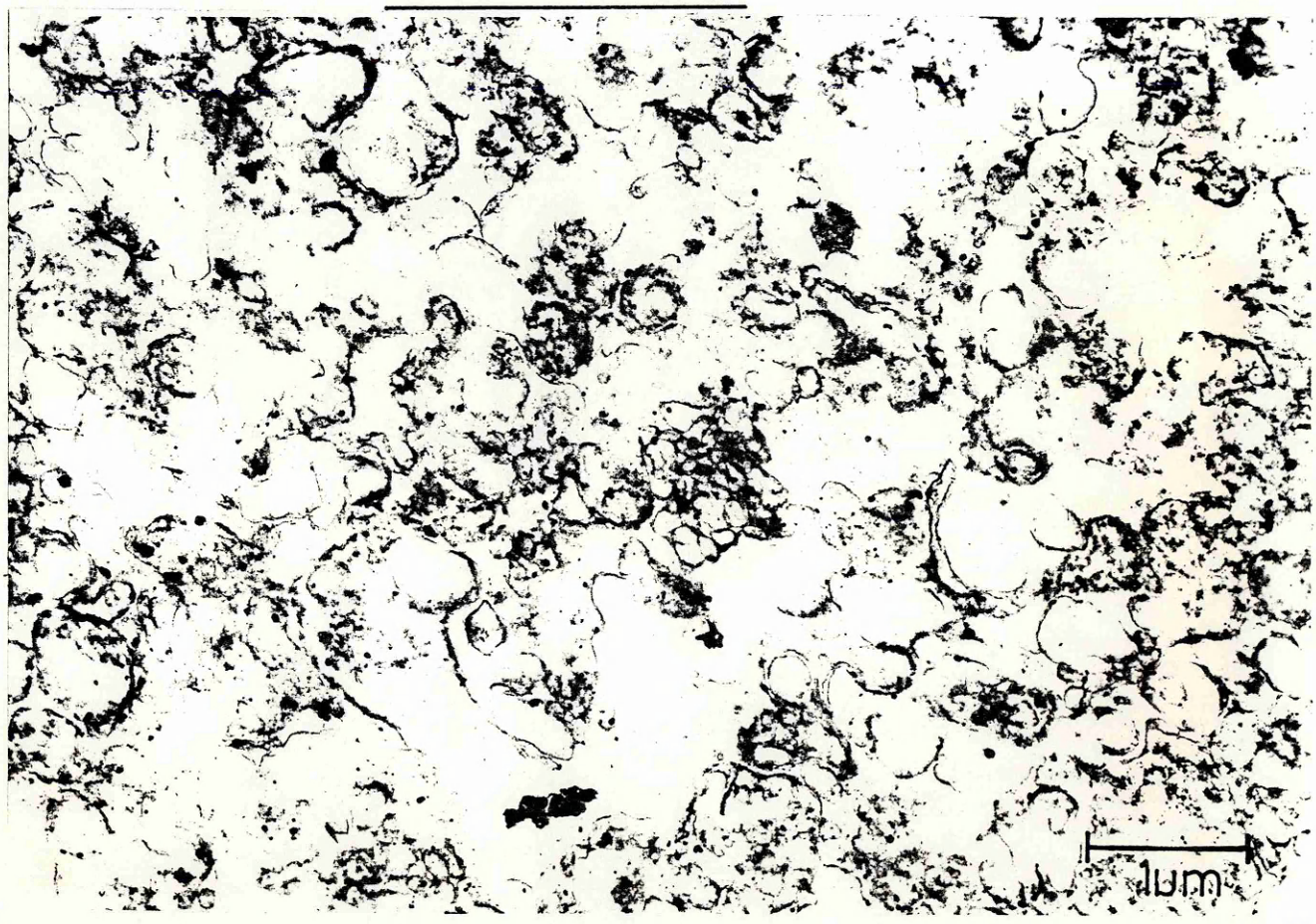


Plate 4 - 50day



Preparation of pure synaptic membranes

The cleaned cortices previously described were homogenized in 9 vols. of 10% (w/w) sucrose using a glass homogenizer with a teflon pestle. The homogenate was centrifuged at 1,000 rpm for 20 mins. and the pellet was discarded. The supernatent was centrifuged at 9,000g for 20 mins. and the supernatent was discarded. The pellet was resuspended in 10% sucrose and recentrifuged at 9,000g for 20 mins. The pellet was dispersed in 2mls. of 5mM Tris HCl pH 8.4 using a pasteur pipette and left on ice for 30 mins. The suspension was then homogenized by hand and 4 mls. of 48% (w/w) sucrose was added to the tube. The contents of the tube were thoroughly mixed and in 15 ml. polycarbonate centrifuge tubes. 5mls of 28.5% (w/w) sucrose and 4 mls. of 10% (w/w) sucrose were then added to the tube and the contents were centrifuged at 60,000 g for 90 mins. The synaptic membranes collected at the interface between the two highest sucrose concentrations and this fraction was carefully removed with a pasteur pipette. The synaptic membrane fraction was made up to 10mls with 50mM potassium phosphate buffer pH 7.1 and centrifuged for 20 mins at 48,000g. The membranes were then resuspended in 50mM potassium phosphate buffer (pH 7.1) for use in the binding assay.

Protein concentrations were estimated by the method of Lowry et al. (1951), using bovine serum albumin as standard.

CHAPTER 4

ESTABLISHING THE BINDING ASSAY

a) Separating bound ligand from free ligand

The theoretical considerations discussed in the previous section provided a basis for studies of the optimum conditions necessary for the investigation of glutamate binding to rat cortical membranes. Earlier reports of glutamate binding in rat cerebral cortex gave estimates of the Kd in the region of 10^{-7}M (Roberts 1974, Michaelis et al.,1974). This indicated centrifugation as the method of choice for separating glutamate bound to brain tissue from the free ligand. However, Roberts (1974) did use filtration in these early studies. Filtration offers considerable advantages over centrifugation in terms of the large number of samples that can be handled, the greater speed at which they can be processed and the ease with which washing procedures can be carried out. Consequently, initial studies attempted to use this technique to separate bound from free ligand. Filtration was carried out using both cellulose filters and glass fibre filters. Unfortunately the filters retained very little of the radiolabel bound to membranes after 3 washes with ice-cold distilled water or 50 mM phosphate buffer. The amount of radiolabel remaining on the filters was equal to the level retained by filters in the absence of membranes

and represented less than .01% of the total radioactivity. Unwashed membranes showed considerable variation between triplicates in the levels of radioactivity adhering to the filters and it was very difficult to systematically show any difference between total binding and non-specific binding. In contrast, centrifugation at 48,000g for 10 mins. at 0°C proved a very effective and reproducible method of separating bound ^{3}H-L-Glu from the free ligand and discriminating between specific and non-specific binding. The concentration range of ^{3}H-L-Glu utilized in the assay was simply arrived at from a consideration of the reported Kd values for glutamate. To test for the effects of other parameters on the binding a single ^{3}H-L-Glu concentration of 0.6μM was routinely used. The concentration of non-radioactive glutamate used in the experimental blank was arrived at from the consideration that it should be at least 100x the Kd value (Burt, 1978) and was routinely at a final concentration of 0.1mM.

b) Incubation Temperature

As glutamate is a ubiquitous biochemical essential for many metabolic processes, it is an enzyme substrate in many reactions. I therefore decided to minimise the risk of such reactions by carrying out the assay at 0°C by incubating the tubes in an ice-water bath. In this way, it was possible to be fairly certain that the radioactivity measured at the end of the assay both bound and free, was indeed glutamate and not another metabolite. These initial studies were carried out using, essentially, a P2 membrane fraction and 0.2 - 0.3 mg was routinely added to each of the assay tubes. The medium for these assays was potassium phosphate buffer at a neutral pH of 7.1. The final volume in each of the tubes was 2 mls. Initially, all estimations were carried out in triplicate, then duplicate tubes were used for pragmatic reasons and because these duplicate values were always within

5% of each other.

c) Estimating the amount of bound ligand

After termination of the assay by rapid centrifugation, an aliquot of the supernatant was taken to estimate free levels of radioactivity and the remaining supernatant carefully decanted. The pellets were then rinsed superficially with 2 x 5mls. of ice-cold distilled water to remove any unbound ^{3}H-L-Glu. Although it is not possible to remove free glutamate trapped inside the pellet, this amount of free ligand will be present in both sets of tubes i.e. those measuring the total binding, containing only radioligand and those measuring nonspecific binding that contain, in addition to the radioligand, excess cold ligand. Thus, subtracting non-specific binding from total binding will automatically solve the problem. After pellet rinsing, the tubes were left to drain at 4°C and the pellet was then solubilized in 1ml. of 0.1M NaOH. Scintillation fluid was added, together with 100μl per vial of glacial acetic acid to eliminate chemiluminescence. Radioactivity was assayed in a Beckman 250 scintillation counter at ambient temperature with a counting efficiency of 30%.

d) Time course of the assay

The time course of the specific binding is shown in Fig. 41. As may be expected, at the low incubation temperature used for the assay, the reaction proceeded slowly, and attained equilibrium only after approximately 10-15 minutes. Non-specific binding on the other hand was essentially instantaneous and remained at the same level throughout. Consequently, an incubation period of 20 minutes was utilized for all binding assays reported in this thesis.

e) The effect of pH

Fig. 4.2 shows the effect of pH on levels of specific binding and demonstrates that there is a broad range of pH values at which binding can be detected (pH4-pH9) although there is maximal binding at around pH7. It was not possible to detect any specific binding below pH4, or above pH9. This is in keeping with the proposed mechanism of interaction between glutamate and its postsynaptic receptor as a 3 point attachment between the two anionic carboxyl groups and the cationic amino group of glutamate and opposite charged groups at the acceptor site, (Curtis & Watkins 1960). As the pk of these three ionizable groups are 2.19, 4.25 and 9.67 respectively, the glutamate molecule will not be in its fully ionized state either below approx pH 4 or above pH9.

f) The effect of protein concentration

Although, wherever possible, I intended to standardize the amount of tissue used per tube in the binding assay, it seemed inevitable that variations in yield would arise, not only on a day to day basis, but also as a result of the use of brain tissue from rats varying in age. Therefore, it was necessary to demonstrate that levels of glutamate binding were directly proportional to the amount of tissue utilized in the bin ing assay. Fig. 4.3 gives the result of such an experiment and shows that specific binding is linear with protein concentrations between 0.1 - 1.0 mg per tube.

g) Subcellular distribution of the binding

One of the main criteria for establishing a substance as a neuro-transmitter is that binding sites capable of specific interaction with the ligand should be concentrated at the site of transmission on the post-synaptic membrane. Thus it is a necessary condition to show that

the specific activity of the binding is enriched in a synaptic membrane preparation over that amount present either in a crude homogenate or in a P2 fraction. Accordingly, I further resolved the P2 pellet by density gradient centrifugation to yield purified Myelin, synaptic membranes and mitochondria according to the method of Jones and Matus (1974) and detailed previously. Specific glutamate binding was then analysed in all fractions i.e. in homogenate (H), S_1, P_2, S_2, Myelin (My) synaptic membranes (SM) and mitochondria (Mit). The results of this study are given in Table 4l and show that the binding is almost exclusively located on a particulate fraction. In addition, there is almost a seven fold increase in the specific activity of the binding in SM compared to that in a P_2 fraction, which would seem to indicate that many of these binding sites are indeed associated with the synapse. Some of the binding, though, is present in the mitochondrial fraction which is contaminated with plasma membranes from extra-synaptic locations. This could be suggestive of the fact that a proportion of the binding sites for glutamate may be located extra-junctionally on the neuronal perikarya.

Fig. 41

Time course of ^{3}H - L - Glutamate binding to cortical membranes. Binding was determined at a (^{3}H-L-Glu) of 0.6μM at 0-2°C. Results are means of 4 separate experiments which varied by less than 10%; specific binding ■ ; non-specific binding □.

Fig. 42

The Effect of pH on the specific binding of ^{3}H-L-Glu to cortical membranes. The results are means of 4 separate experiments which differed by less than 5%. Assay conditions as stated in the text.

FIG 41

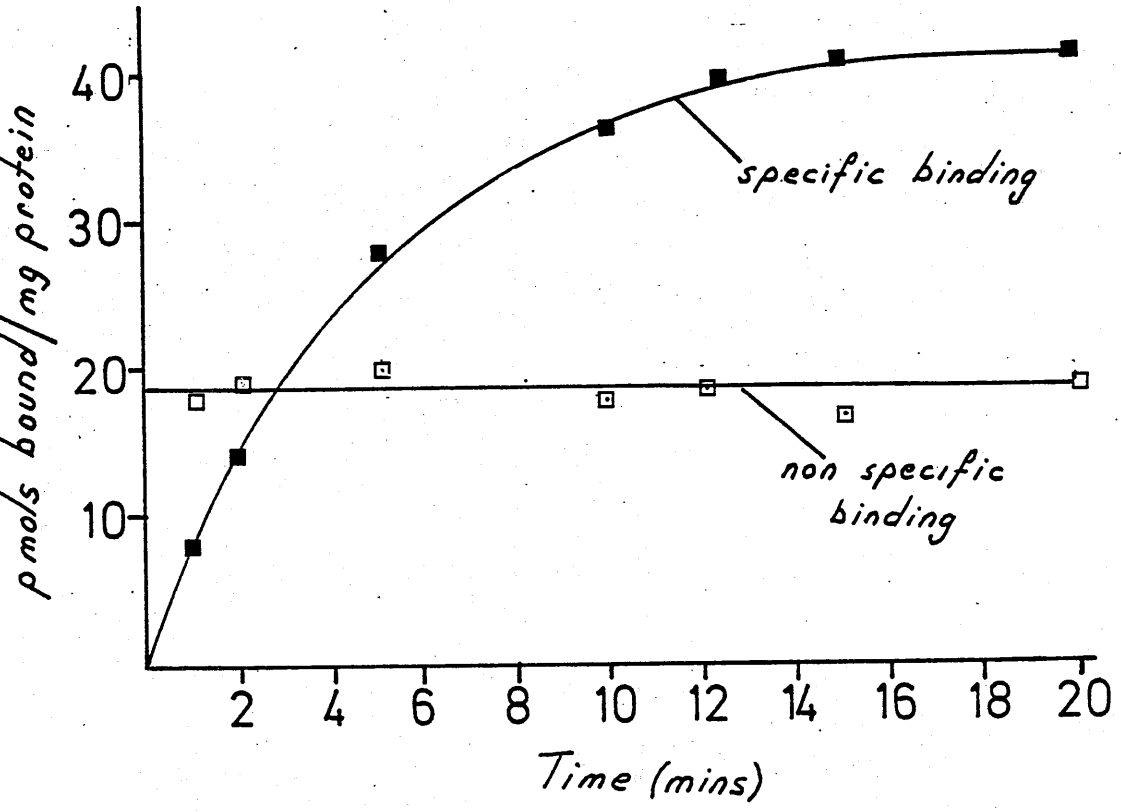

p mols bound/mg protein
40
30
20
10
specific binding
non specific binding
2
4
6
8
10
12
14
16
18
20
Time (mins)

FIG 42

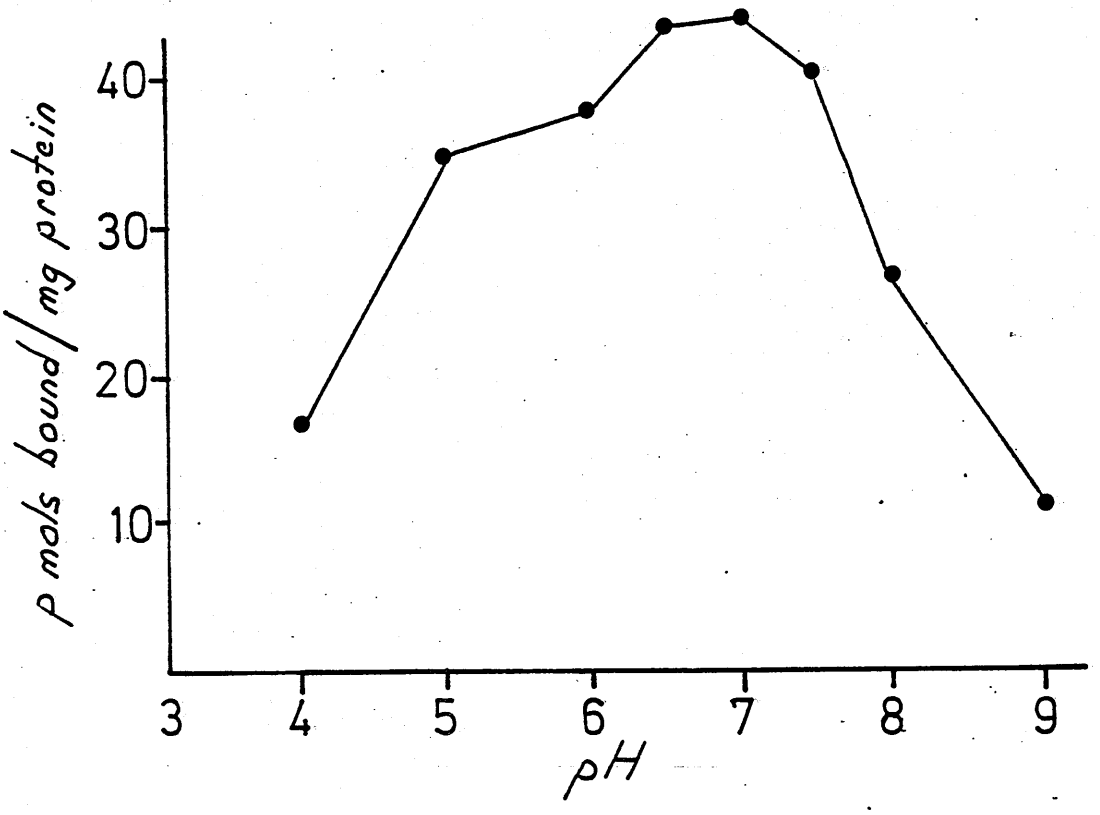

p mols bound/mg protein
40
30
20
10
3
4
5
6
7
8
9
pH

Fig. 43

The effect of increasing amounts of cortical membranes on the specific binding of ^{3}H-L-Glu. Tissue is estimated as mg protein per assay tube. Results are means of 4 -6 separate experiments which differed by less than 10%.

FIG 43

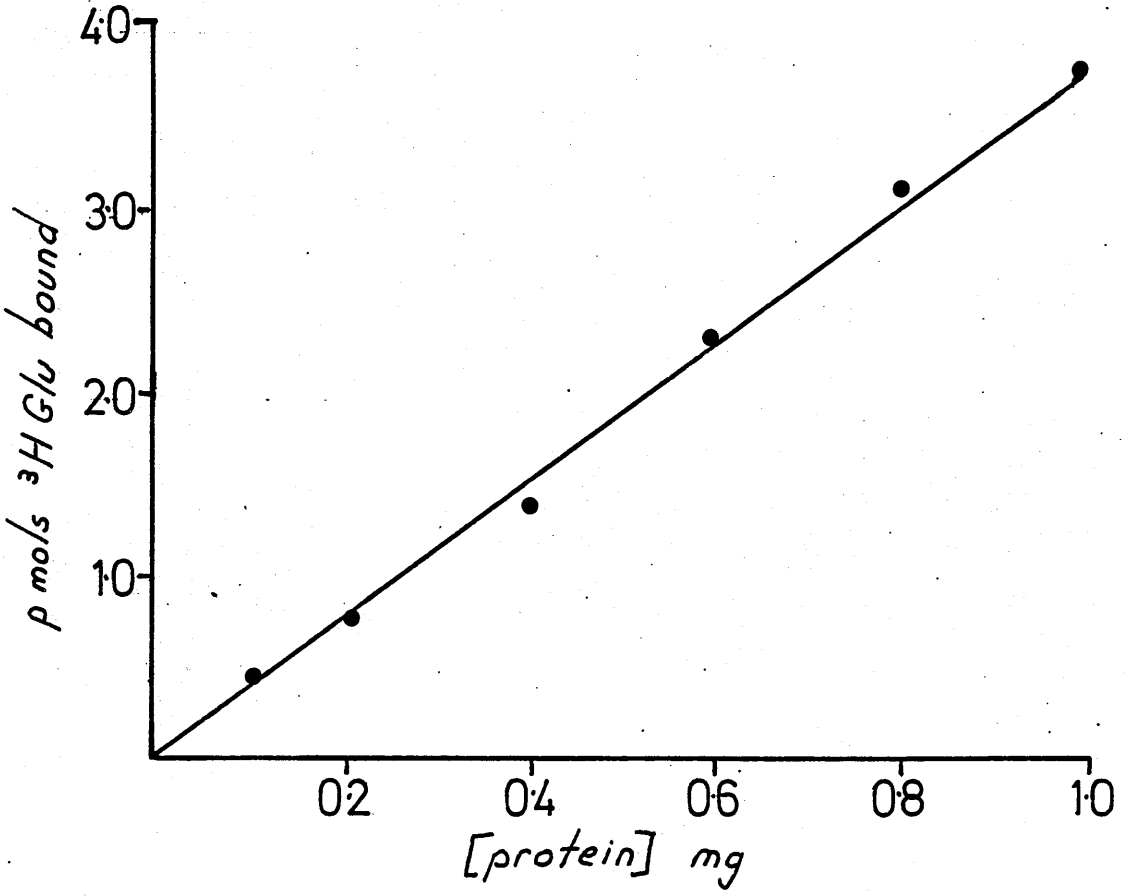


TABLE 41 Distribution of glutamate binding in different subcellular fractions.

Fraction	Specific Binding (p mols/mg protein) K (P) Buffer	Na(P) Buffer
H	2.75 ± 0.14	25.4 ± 0.8
S_1	3.75 ± 0.16	24.2 ± 1.1
P_2	4.5 ± 0.21	46.8 ± 1.8
S_2	0.2 ± 0.018	1.4 ± 0.2
My	0.0	0.0
SM	31.0 ± 2.4	84.0 ± 7.2
Mit	3.2 ± 0.24	35.1 ± 4.1

Synaptic membranes were prepared as previously described and results are means ± S.E.M. of six experiments.

h) The Effect of Sodium

Table 4 also shows the results of a similar experiment but, where the K(P) buffer used as the incubation medium in the assay was replaced with Na(P) buffer at the same pH of 7.1. This was in an attempt to recognise binding to transport sites for glutamate that may serve a transmitter function and that are highly dependent on the presence of Na^+. Once again, activity is located solely in the particulate fraction. The binding, however, is not strongly associated with the synaptic membrane fraction for, although there is a two fold increase in activity in this fraction over the P_2 pellet, there is also a significant proportion of the binding in the mitochondrial fraction. Many of these putative high affinity transport sites are thought to exist on glial cell surfaces, and the plasma membrane of these cells will be present in the mitochondrial pellet as well as being a contaminant of the synaptic fraction. Levels of binding in the presence of sodium are approximately 6 x the level of binding demonstrable in the absence of sodium.

i) The dose response to potassium (K^+)

Potassium phosphate buffer was chosen as the incubation medium because, in contrast to other workers (e.g. Roberts, 1974), I could not detect any specific binding of glutamate using either 50mM Tris. citrate buffer (pH 7.1) or 50 mM Tris. HCl buffer (pH 7.1). Fig. 4.4 shows the effect of increasing concentrations of potassium on specific glutamate binding. The incubation medium used in the assay was Tris. citrate buffer and the potassium was added as the chloride salt to control for any effect of increasing phosphate concentrations on the binding. As can be seen from the graph the binding seems to be quite markedly dependent

on the presence of potassium and reaches maximal levels at about 20 mM K^+ when the p/mols of glutamate specifically bound per mg protein is very similar as in the presence of 50mM potassium phosphate buffer. This result is in stark contrast to studies performed by Sharif and Roberts on the cerebellum where they reported a marked inhibition of glutamate (and aspartate) binding by K^+ over a concentration range of 2-50mM K^+ (Sharif and Roberts, 1981). The differences in the two sets of results are difficult to explain, but may possibly represent a regional variation in the modifying effects of cations.

An alternative explanation may be provided by more recent studies that report an enhancing effect of Chloride ions on L-glutamate binding to rat brain synaptic membranes (Mena et al 1982). As the potassium concentration in the incubation medium in my own studies was altered by the addition of the chloride salt it seems likely in the light of this recent work that the data shown in Fig. 4.4 reflects this dose dependent enhancement of glutamate binding by chloride ions, rather than a direct effect of potassium ions. The characteristics of the chloride enhancement show strong similarities to the effect on glutamate binding shown in Fig. 4.4 in that the binding was saturable over the same chloride concentration range reaching a maximal at approx. 20mM Cl^-. The possible effect of ions on glutamate binding will be discussed further in the final chapter of this thesis.

j) <u>The dose response to sodium</u> (Na^+)

This view is further strengthened from a consideration of the effects of sodium in the two preparations. Fig. 4.5 shows a biphasic response to sodium, with low concentrations producing a dose dependent inhibition of the binding. Higher concentrations produce a large increase in observed levels of specific glutamate binding. The change

Fig. 44

The effect of increasing ionic concentrations of K^+ on the specific binding of ^{3}H-L-Glu. The tissue was incubated under conditions described in the text in 50mM. Tris-Citrate buffer to which varying concentrations of K^+ were added as the chloride salt. The results are means of 5 separate experiments which differed by less than 5%.

Fig. 45

The effect of increasing ionic concentration of Na^+ on the specific binding of ^{3}H-L-Glu. Na^+ was again added as the chloride salt. The results are means of 4 separate experiments which differed by less than 10%.

FIG 44

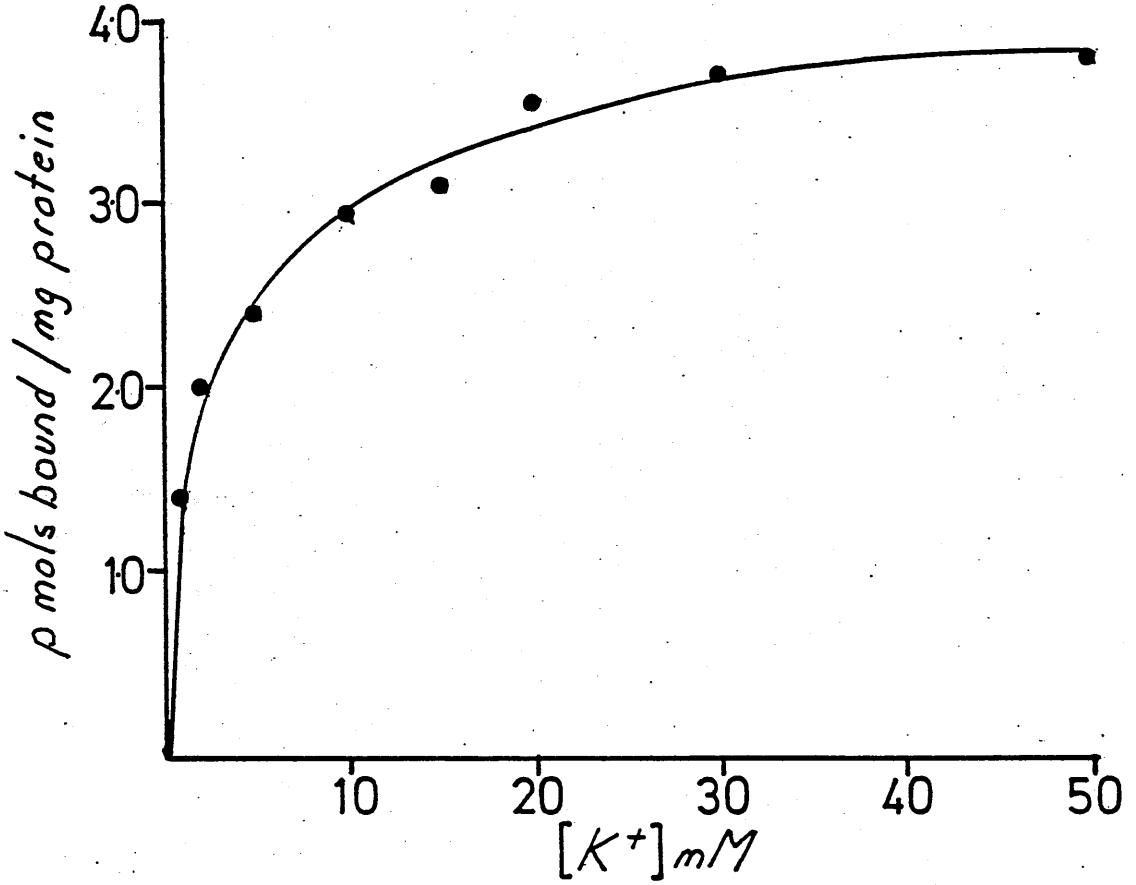
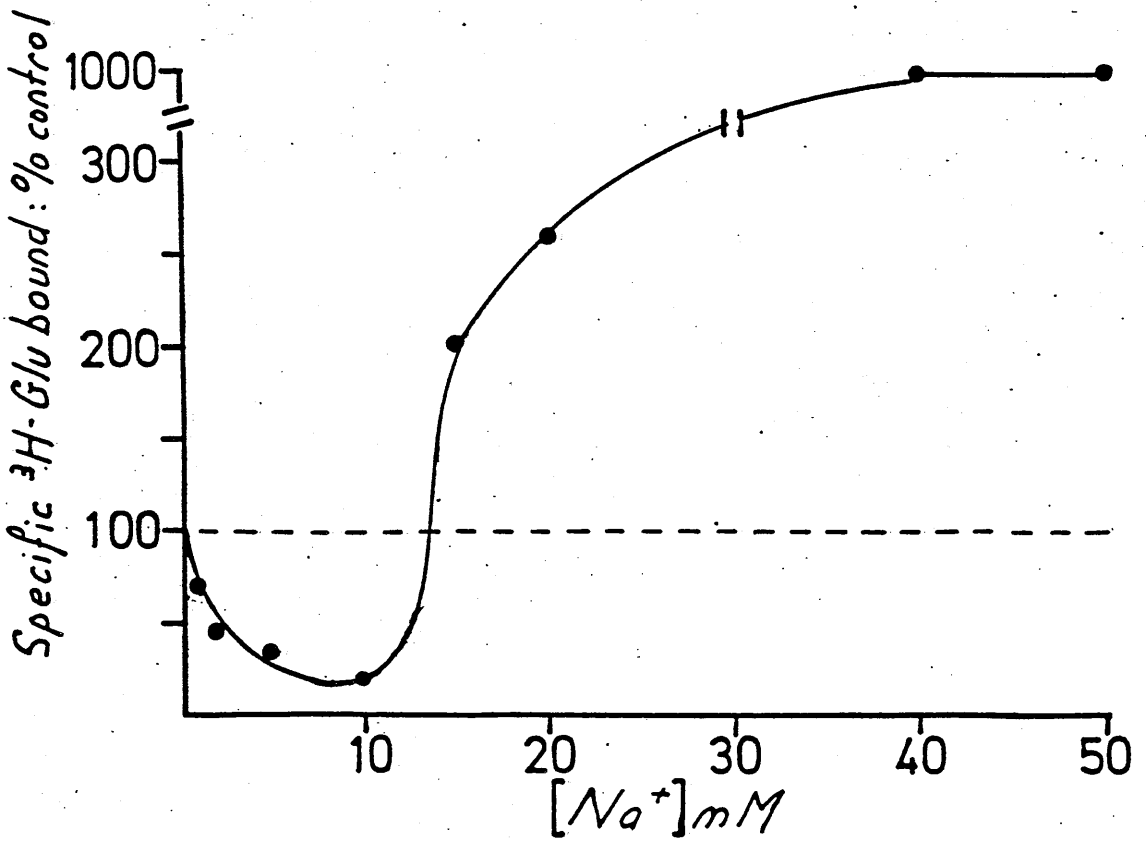

40
30
20
10
p mols bound / mg protein
10
20
30
40
50
[K+] mM

FIG 45


1000
300
200
100
Specific ³H-Glu bound : % control
10
20
30
40
50
[Na+] mM

from inhibition to stimulation of binding occurs at a Na^+ concentration of about 10mM. This effect of sodium parallels the situation found in hippocampus (Baudry and Lynch, 1979; Vargas and Costa, 1981), but is in contrast to the reported effects of Na^+ in the cerebellum where an inhibition of binding is apparent with sodium concentrations up to 100mM (Foster and Roberts 1978; Sharif and Roberts, 1981). In addition, several workers have reported binding sites on cortical tissue that are sodium dependent and that can be distinguished from the sodium independent binding component on the basis of kinetic parameters (Roberts, 1974).

k) Tissue distribution of binding sites

If the specific sodium independent binding of glutamate is attributable to interaction with a postsynaptic receptor then it may be expected that such sites would be limited to the nervous system. Accordingly I investigated levels of Na^+ independent glutamate binding to P_2 fractions prepared from whole brain, heart, kidney, liver, lung and peripheral (skeletal) muscle, as well as from cerebral cortex. No binding was detectable in either the kidney, liver or lung. Binding to the whole brain was slightly lower than in the cortex. However, surprisingly and curiously, levels of specific binding were very high in skeletal muscle and in heart muscle. The relative proportion of binding that was specific compared to non-specific fell to approximately 20% in these tissues however. These results are summarised in Table 42. It is rather difficult to assign a function to these binding sites in heart and striate muscle, for although both tissues are extensively innervated, glutamate has not been shown to be the transmitter utilised by the peripheral and autonomic nervous system.

TABLE 42 DISTRIBUTION OF GLUTAMATE BINDING BETWEEN DIFFERENT TISSUES

Tissue	Binding (p mols/mg protein) Specific Binding	Non-specific binding
Cortex	3.8 ± 0.14	2.5 ± 0.13
Whole Brain	2.75 ± 0.12	2.4 ± 0.13
Heart	7.4 ± 0.36	28.6 ± 2.1
Kidney	0.0	-
Liver	0.0	-
Lung	0.0	-
Skeletal Muscle (from upper hind leg)	19.4 ± 1.1	74.6 ± 2.8

Sharif and Roberts (1981) have reported that a 3 minute pre-incubation of membranes prepared from cortical tissue at 37°C causes an enhancement of specific glutamate binding of over 400%. They attribute this finding to the presence of an endogenous inhibitor of glutamate binding which is released uring the pre-incubation step and is subsequently washed out. Accordingly, I looked at the effect of such a pre-incubation on the membrane fraction used in the binding assays reported here. Freshly prepared membranes, ready for use in the binding assay, did not seem to be affected by this treatment, although the experiment was repeated several times varying the number of subsequent washes from 2 - 6. It may be that the routine step of suspending membranes for 30 minutes at 0°C in hypotonic buffer (5mM Tris HCl pH 8.4) serves the same function as the 37°C incubation in the study by Sharif and Roberts, i.e. the releasing and washing out of endogenous ligands. An analysis of the wash supernatent in an attempt to identify possible modulators may prove fruitful. Levels of binding were not significantly increased by subsequent washes of the pellet to be used in the assay over and above the two utilized routinely. Similarly, further washes of the pellet at the termination of the assay did not decrease levels of radioactivity.

In summary, the binding assay was routinely performed as stated below:

Tissue sample:	0.3 - 0.6 mg protein, routinely P_2 fraction.
Buffer:	50mM potassium or sodium phosphate, pH 7.1.
^{3}H-L-Glu:	0.005 - 2.0 mM (0.2 - 0.4 nCi/tube approx.)
'Cold' L?Glu:	0.1mM.
Total Tube vol:	2 mls.
Incubation:	20 mins at 0°C.
Termination:	Centrifugation at 48,000g for 10 min.

CHAPTER 5

RESULTS

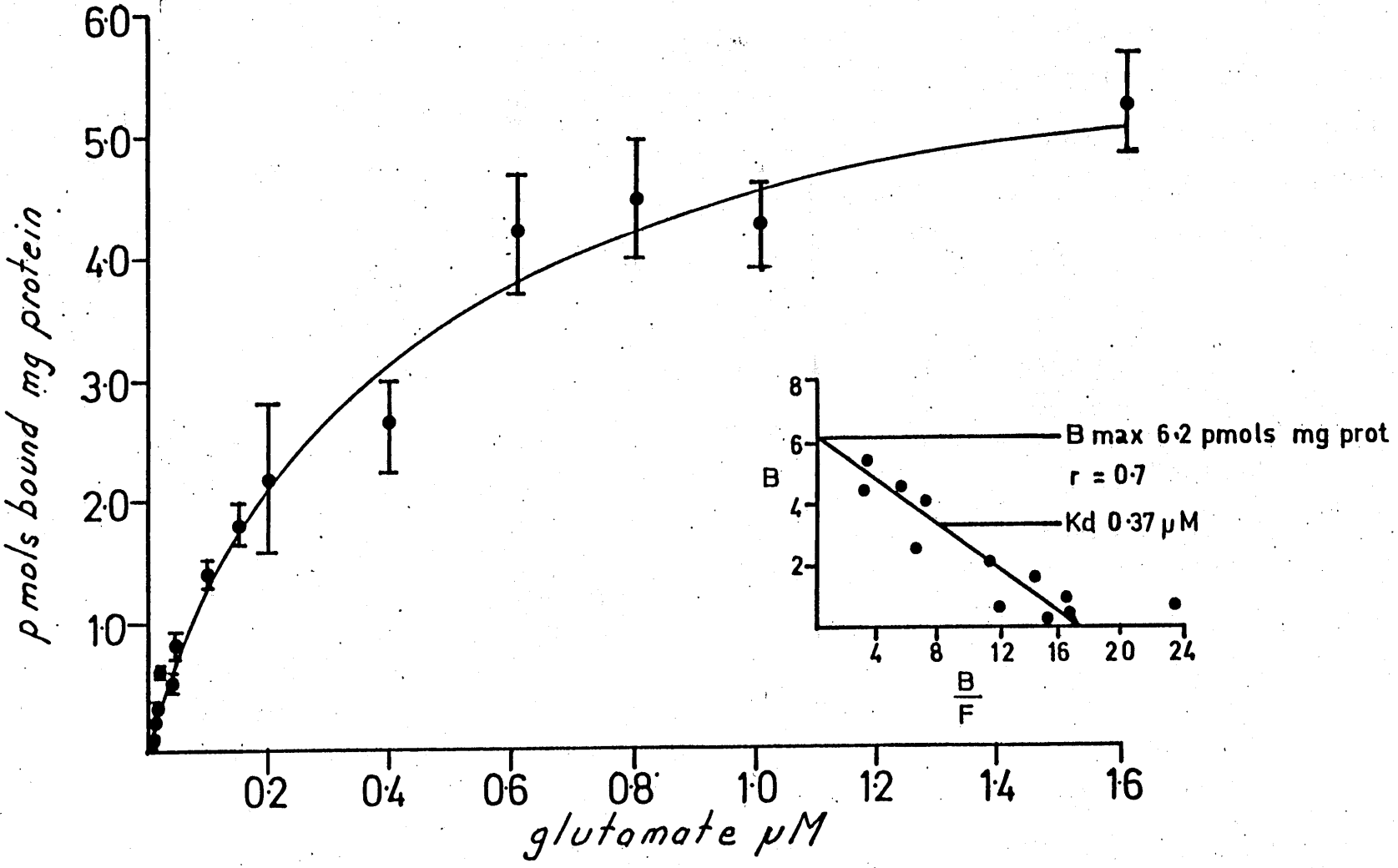
5.1 Glutamate Binding to Adult Rat Cortical Membranes

Figure 5.1 shows the binding of ^{3}H-L-Glu to cortical membranes prepared from 50 day old rats, and carried out in the absence of Na^{+}. Specific binding is saturable over the concentration range of 0.005-1.6μM but non specific binding was not saturable over the same concentration range, and increased linearly ($r = 0.99$). Replotting this data by Scatchard analysis (Inset 5.1) shows this site to have an apparent dissociation constant (K_d) of $0.37 \pm 0.06 \mu m$ with a site density (B_{max}) of 5.20 ± 0.57 pmols/mg protein. Standard errors of the mean of kd and Bmax were calculated throughout this thesis by grouping together the kinetic values from at least five individual experiments. In view of recent reports (Biziere et al, 1980; Roberts and Sharif, 1981) of very high affinity binding sites for glutamate on membranes prepared from forebrain

Fig. 51

The specific Na^+ independent binding of ^{3}H-L-Glutamate to cortical membranes (P_2) prepared from adult (50 day old) rats. ^{3}H-L-Glu concentrations varied between 5×10^{-9} and 1.6×10^{-6}M. The results shown are the mean $\pm$ SEM of 6-8 separate experiments. Kinetic paramaters were estimated from a Scatchard plot of the data which is shown as an insert. The line on the Scatchard plot was drawn by linear regression analysis.

FIG 5₁


6·0
5·0
4·0
3·0
2·0
1·0
p mols bound mg protein
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
glutomate μM
8
6
4
2
B
B max 6·2 pmols mg prot
r = 0·7
Kd 0·37 μM
4
8
12
16
20
24
B/F

and striatum respectively with Kds of 11nM (Biziere et al - forebrain) and 16.6 nM (Roberts - striatum) the initial part of the saturation isotherm (from 5nM - 100μ M) was analysed by linear regression. A plot of f mol bound/mg protein vs ^{3}H-L-Glu gives a coefficient of determination of the slope of the line of 0.9, showing that the binding over this concentration range represented the initial linear part of the total binding isotherm and did not represent a separate saturation curve.

Figure 5.2 shows the binding of 3-H-L-Glu in the presence of 50mM Na^{+}. Specific binding is again saturable over the concentration range, while non-specific binding was linear. A Scatchard analysis of the data reveals a Kd of 1.34 $\pm$ 0.16μM and a Bmax of 210 $\pm$ 19.20 pmols bound/mg protein (Insert Figure 5.2).

Discussion

The presence of two high affinity binding sites for L-glutamate on cortical membranes fits well with currently accepted criteria for neurotransmitter status. All amino acid candidates so far examined appear to be taken up into nerve endings by both a low affinity (Kd = 10^{-3}M) and a high affinity (Kd = 10^{-5} - 10^{-6}M) transport system in contrast to other amino acids which are only transported by a low affinity system. These high affinity sites have a strict requirement for sodium and have been proposed for sometime as the mechanism by which neurotransmitter activity is terminated (Roberts, 1974; Bennett et al, 1973; Iversen, 1971). Since then, there have been reports of Na^{+} dependent binding sites in many brain areas. These are summarised in Table 5.1. In addition it has been shown that sodium is necessary for the initial binding event (Wheeler and Hollingsworth, 1978) and so in vitro studies on the interaction of

Fig. 52

The specific Na^+ dependent binding of ^{3}H-L-glutamate to cortical membranes (P_2) prepared from adult (50 day old rats) ^{3}H-L-Glu concentrations varied between 0.1 - 1.6 μM. The results are means $\pm$ SEM of 8 separate experiments. kinetic parameters were estimated from a Scatchard plot of the data which is shown as an inset.

FIG 52

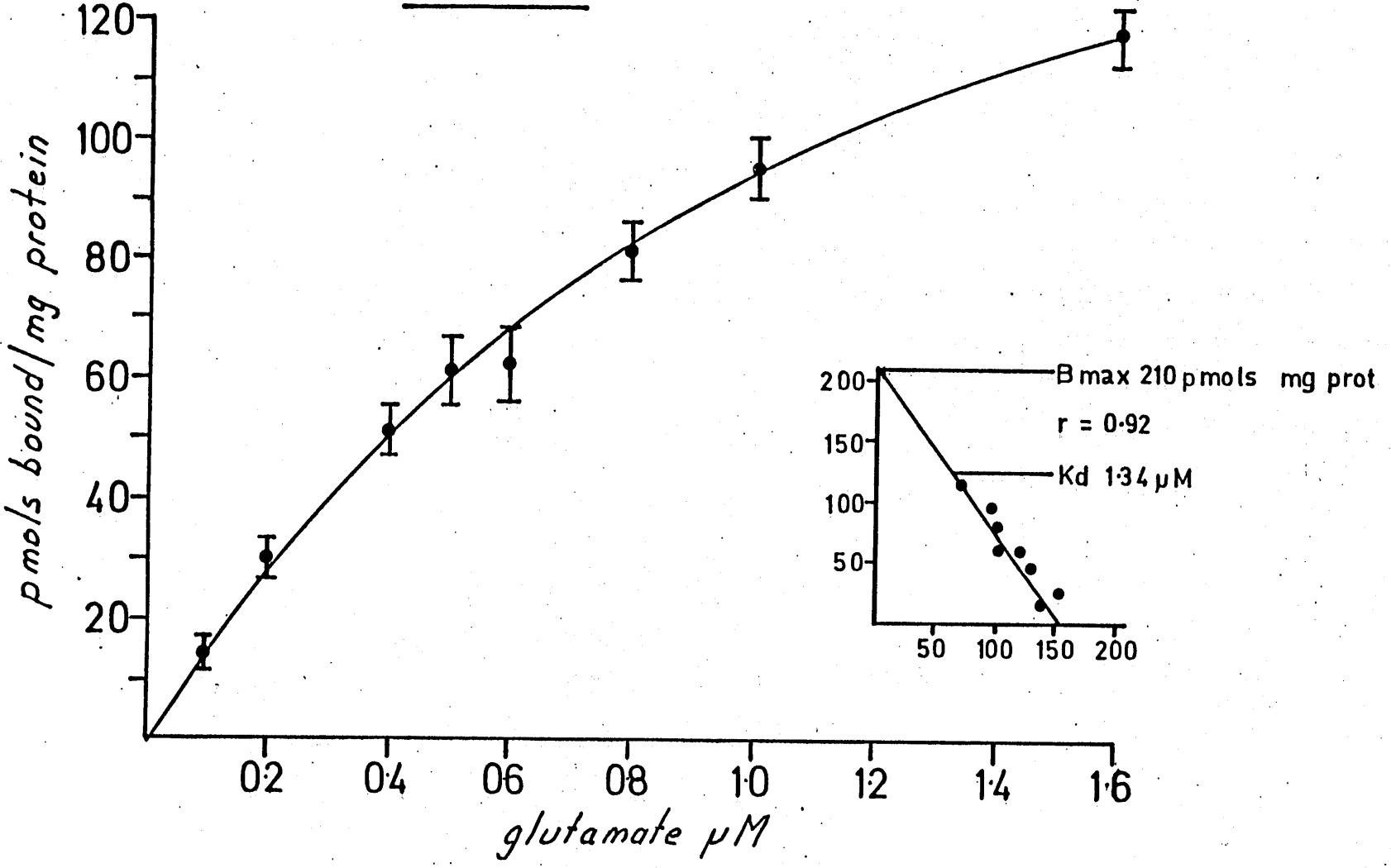

120
100
80
60
40
20
pmols bound/mg protein
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
glutamate μM
200
150
100
50
50
100
150
200
B max 210 pmols mg prot
r = 0·92
Kd 1·34 μM

TABLE 5 Kinetic values of specific glutamate binding in different areas of rat brain

Rat brain area	Method	Bmax (pmol.mg.prot $^{-1}$)	Kd μM	Reference
Whole brain	Filtration 0.1 - 30μM	4440	0.18 2.08	Michaelis et al (1974)
Whole brain	centrifugation 0.001-0.7 μM	(a) 0.13 (no Na^+) (b) 1.16 (no Na^+)	0.01 0.08	Biziere et al (1980)
Forebrain	centrifugation 0.050 - 0.75 μM	61.6 (no Na^+)	0.83	Foster et al (1981)
Cerebellum	centrifugation 0.001-1.8 μM	73 (no Na^+)	0.74	Foster and Roberts (1978)
*Cerebellum	centrifugation	2.45 (no Na^+) 1.39 (no Na^+)	0.37 (adult) 0.39 (14 day)	Slevin and Coyle (1981)
Cerebellum	centrifugation 0.007-1.85 μM	54.5 (no Na^+) 13.0 (no Na^+)	0.82 0.51	Honore et al (1981)
Striatum	1 - 5 μM	336.2 (Na^+)	3.46	Vincent and McGeer (1980)
Hippocampus	filtration	4.5 (no Na^+)	0.66	Baudry and Lynch (1979)
Hippocampus	filtration 0.1-10 μM	6.5 (no Na^+) 75 (Na^+)	0.75 2.4	Baudry and Lynch (1981)
*Hippocampus	filtration	6.5 (no Na^+) 7-10 (no Na^+)	0.43 (adult) 0.43 (neonate)	Baudry et al (1981)

TABLE 5₁ (Cont'd...)

Rat brain area	Method	Bmax (pmol.mg.prot^{-1})	Kd μM	Reference
Cerebral cortex	0.06-250 M	(a) 530 (b) 32000 (c) 166000	0.3 5.0 55	De Robertis and Fiszer de Plazas (1976)
Cerebral cortex	Filtration	(a) 200 (b) 28	4.0 8.3	Roberts (1974)
*Cerebral cortex	Centrifugation 0.005-1.6 M	(a) 210 (Na^+) (b) 8.4 (No Na^+)	1.34 0.37 1.32 (20 day)	Sanderson and Murphy (1982)

* Developmental study

glutamate with its reuptake site should show a strict dependence on Na^+. In view of the estimated affinity constant for this interaction being very similar to the affinity constant for the transport of glutamate by the high affinity process, together with the fairly high site density, there seems a high probability that the binding demonstratable in the presence of Na^+ is indeed to such an uptake site. Kinetic determinations alone, however, cannot be conclusive. Na^+ independent binding may contribute up to 5% of the Na^+ dependent binding, although there have been reports that Na^+ independent binding is greatly inhibited in the presence of low concentrations of $Na^+ < 10mM$ (Baudry and Lynch, 1979; Sharif and Roberts, 1981). Although the maximum degree of this inhibition was difficult to assess at concentrations of Na^+ in excess of 10 mM due to the appearance of Na^+ dependent binding, it may be that it is not possible to measure binding simultaneously to uptake and putative receptor sites.

It is much more difficult to estimate, on the basis of the kinetic evidence only, whether or not the Na^+ independent site is synonymous with a postsynaptic receptor site. Reports of such binding by other workers cover a wide range of kinetic parameters (Table 5.1) and in agreement with Roberts (1981) it seems unlikely that they could all represent binding to a physiologically significant postsynaptic receptor. It is probable that the wide range of reported values is due to the different experimental methodology employed by various workers. Considered within the context of the two-state model of receptor theory previously discussed (Chapter 2) such differences could lead to widely differing ratios of receptor in the R and T states. Variation between different brain regions may reflect real differences in physiological function. Other differences, however, may be due to experimental variables such as the use of filtration as a separation method as opposed to centrifugation

(see section 2.3 for discussion) or to the use of frozen tissue in preference to freshly prepared samples. Foster and Roberts (1978) made the initial observation that glutamate binding, at least in the cerebellum, was very sensitive to denaturation at low temperatures. Later reports from the same laboratory (Sharif and Robert, 1981) have shown that lyophilization of freshly prepared membranes considerably enhances the binding, increasing the apparent Bmax. My own work (reported more fully in a later section) has shown that Na^+ independent and Na^+ dependent binding are differentially affected by freezing and thawing, although both exhibit different levels of binding to that found in fresh tissue. Other methodological differences that may contribute to anomalous kinetics include the purity of the membrane fraction used, the presence or absence of certain cations recently reported to affect glutamate binding (Baudry and Lynch, 1981), the specific activity of the radioligand utilized in the study, the presence of endogenous ligand, and many others. Clearly then there are many important factors that ought to be considered and controlled for whenever possible (as discussed in Section 2.3).

Despite the methodological problems there are indications that Na^+ independent binding, as it has so far been characterized, is at least consistent with binding to a postsynaptic receptor. In particular, site densities are low and there is a considerable enrichment (up to eight times) of the binding in a synaptic membrane fraction as opposed to the homogenate. (Chapter 4).

5.2 Ontogeny of Glutamate binding sites in rat cortex.

Having established the presence of binding sites for glutamate in the adult, the development of these binding sites from birth to 50 days was then investigated. This was motivated by three main questions:-

1) If the binding sites represent biochemical parameters mediating synaptic transmission of glutamatergic neurons, is their development co-ordinated or are there at least correlations between their patterns of development?

2) Do the binding sites appear in the mature form (i.e. exhibiting similar kinetic parameters as in the adult) at an early age or are there qualitative or quantitative changes?

3) Do the developmental profiles exhibit stages or discontinuities that may be correlated with specific morphological events?

The dissection preparation and subsequent handling of cortical membranes from neonatal and very young (up to 10 day old) rats proved problematic and tissue samples used to estimate levels of glutamate binding in rats age 0-10 days were pooled from littermates.

The ontogenetic profile of the Na^+ independent site is shown in Figure 5.3 and that of the Na^+ dependent site in Figure 5.4. The binding is expressed both in terms of pmol bound per cortex and per mg protein. This reflects a primary interest in estimating changes in the total number of binding sites during development. A change in p mols bound/mg protein during this time could simply be a reflection of variation in the relative amount of protein in the brain rather than a change in the absolute number of binding sites. The specific activity of the binding throughout development in terms of p mols bound/mg protein is shown as an inset on both graphs. In view of the complexity of brain maturation it is difficult to interpret these inset graphs in terms of morphological events, although it is an interesting observation that there is a dramatic fall in specific activity of the binding in both graphs at around 15 days. This could reflect

FIG. 53

Developmental profile of specific Na^+ independent ^{3}H-L-Glu binding to cortical membranes prepared from rats of various ages as shown. Binding was estimated at a ^{3}H-L-Glu concentration of 0.6μM. Each point represents the mean $\pm$ SEM of 4 - 7 separate determination. The inset shows the specific activity of the binding in terms of p mols specific ^{3}H-L-Glu bound/mg protein.

FIG 5₃

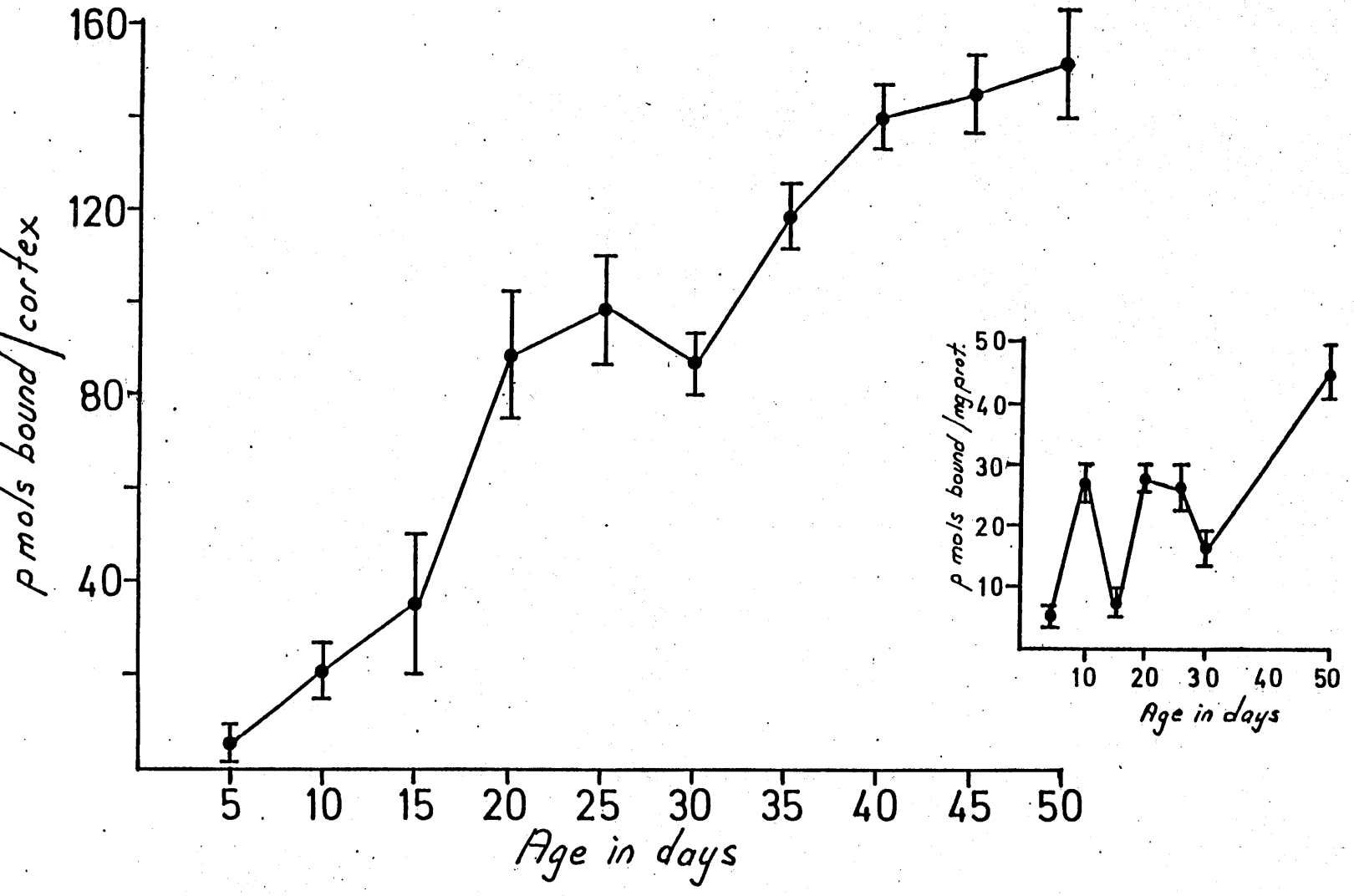
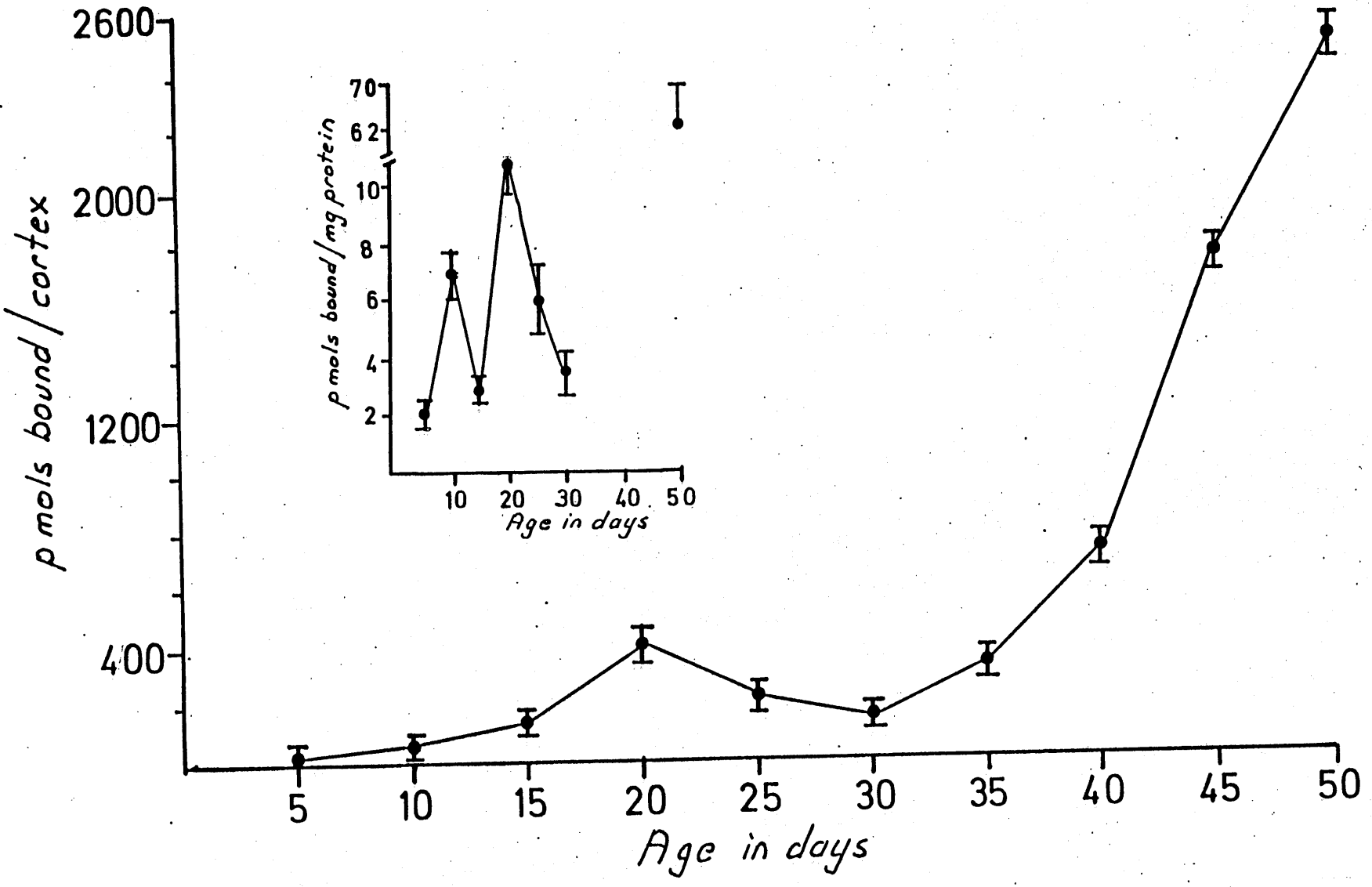

160
120
80
40
p mols bound/cortex
5
10
15
20
25
30
35
40
45
50
Age in days
p mols bound/mg prot.
50
40
30
20
10
10
20
30
40
50
Age in days

FIG. 54

Developmental profile of the specific Na^+ dependent binding of ^{3}H-L-Glu (measured at a concentration of 0.6 μM) to cortical membranes prepared from rats of various ages as shown. Each point represents the mean $\pm$ SEM of 4 - 7 separate determinations.
The inset shows the specific activity of the binding in terms of pmols specific ^{3}H-L-Glu bound mg protein.

FIG 54


2600
2000
1200
400
p mols bound/cortex
5
10
15
20
25
30
35
40
45
50
Age in days
70
62
10
8
6
4
2
p mols bound/mg protein
10
20
30
40
50
Age in days

both the rapid proliferation of neuropil material beginning at around this time and the end of a dramatic phase of proliferation of the dendritic tree, expansion of some size and development of an extensive rough endoplasmic reticulum, indicative of enhanced protein synthetic capacity.

Expressed in terms of p mols bound/cortex, both binding sites were demonstrable from about 5 days of age, albeit at very low levels. Failure to detect binding from 0-5 days may be due to the difficulty of adapting the dissection and fractionation procedure to cope with small amounts of immature cortical tissue rather than an absence of binding per se.

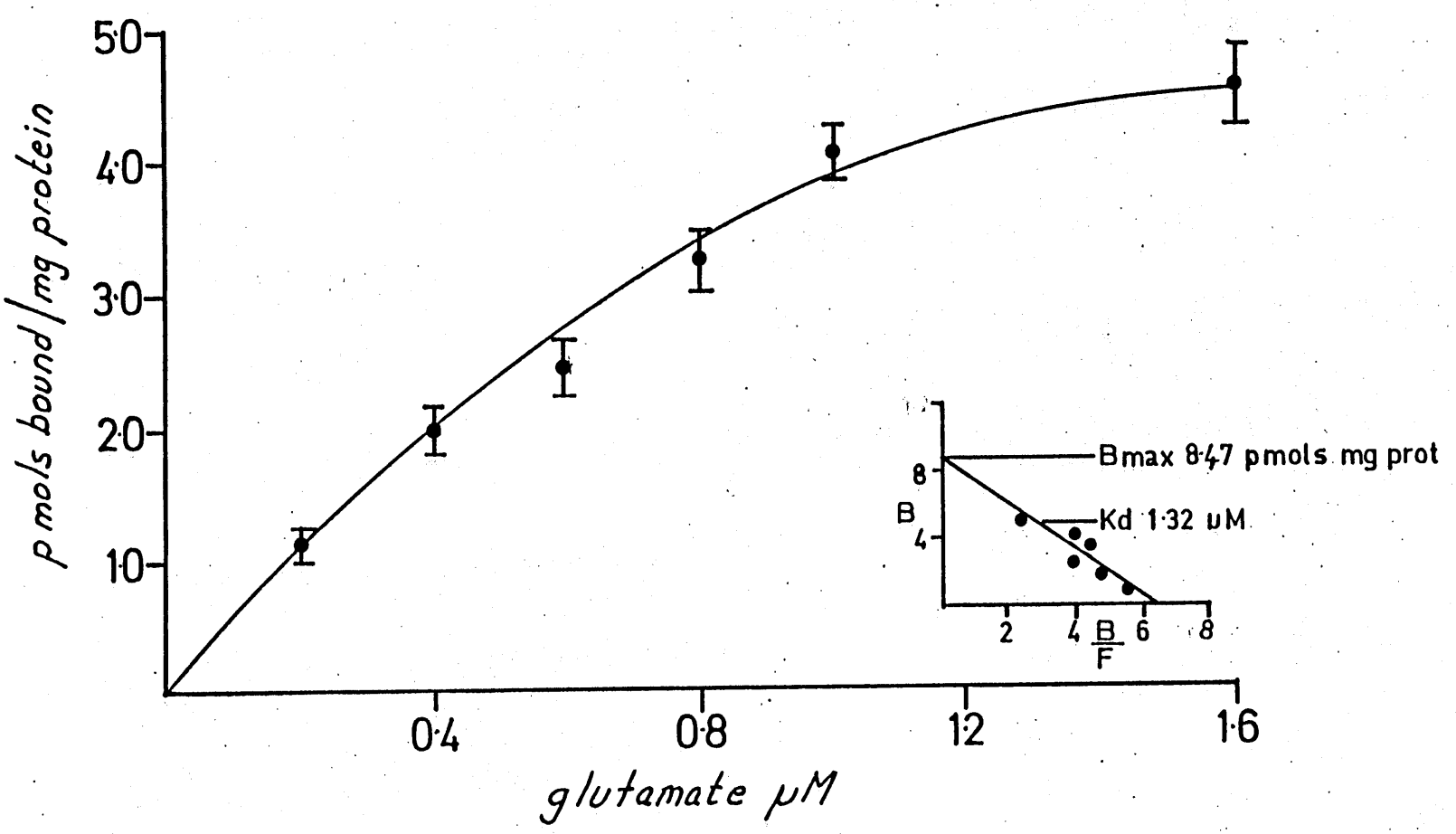
Na^+ independent binding rises sharply to 20-25 days and then more gradually to reach adult levels at 50 days. Na^+ dependent binding on the other hand rises slowly to reach a peak at 20 days dropping significantly ($p < 0.01$) over the next 10 days, before rising again (6 - fold) to reach adult levels at 50 days.

The 20 day age point seems to represent a recognizable stage in the development of glutamate binding sites and this age also corresponds with the time at which maximum rates of synaptogenesis are occurring in the cortex (Agahajanian and Bloom, 1967). Consequently, the kinetic parameters defining glutamate binding of this stage were examined. Figure 5.5 shows a saturation curve for Na^+ independent glutamate binding to cortical membranes prepared from 20 day old rats. The binding does indeed saturate over the same concentration range as in the adult and a Scatchard plot of the data, shown as an inset, gives a Kd of $1.32 \pm 0.23\ \mu M$ and a Bmax of 8.47 ± 1.05 p mols/mg protein.

FIG. 55

The specific Na^+ independent binding of ^{3}H-L-Glu to cortical membranes prepared from 20 day old rats measured at a L-Glu concentration of 0.6 μM. The results show the mean $\pm$ SEM of 6 separate experiments. The kinetic parameters were estimated from a Scatchard plot of the data which is shown as an inset on the graph.

FIG 5₅


5·0
4·0
3·0
2·0
1·0
p mols bound/mg protein
0·4
0·8
1·2
1·6
glutamate μM
Bmax 8·47 pmols mg prot
Kd 1·32 uM
8
B
4
2
4
B/F
6
8

The saturation curve for Na^+ dependent binding is shown in Figure 5.6. Replotting the data as a Scatchard plot gives an estimate for Kd of $1.77 \pm 0.30 \mu M$ and a Bmax of 82.4 ± 4.19 p mols/mg protein (inset Figure 5.6).

Discussion

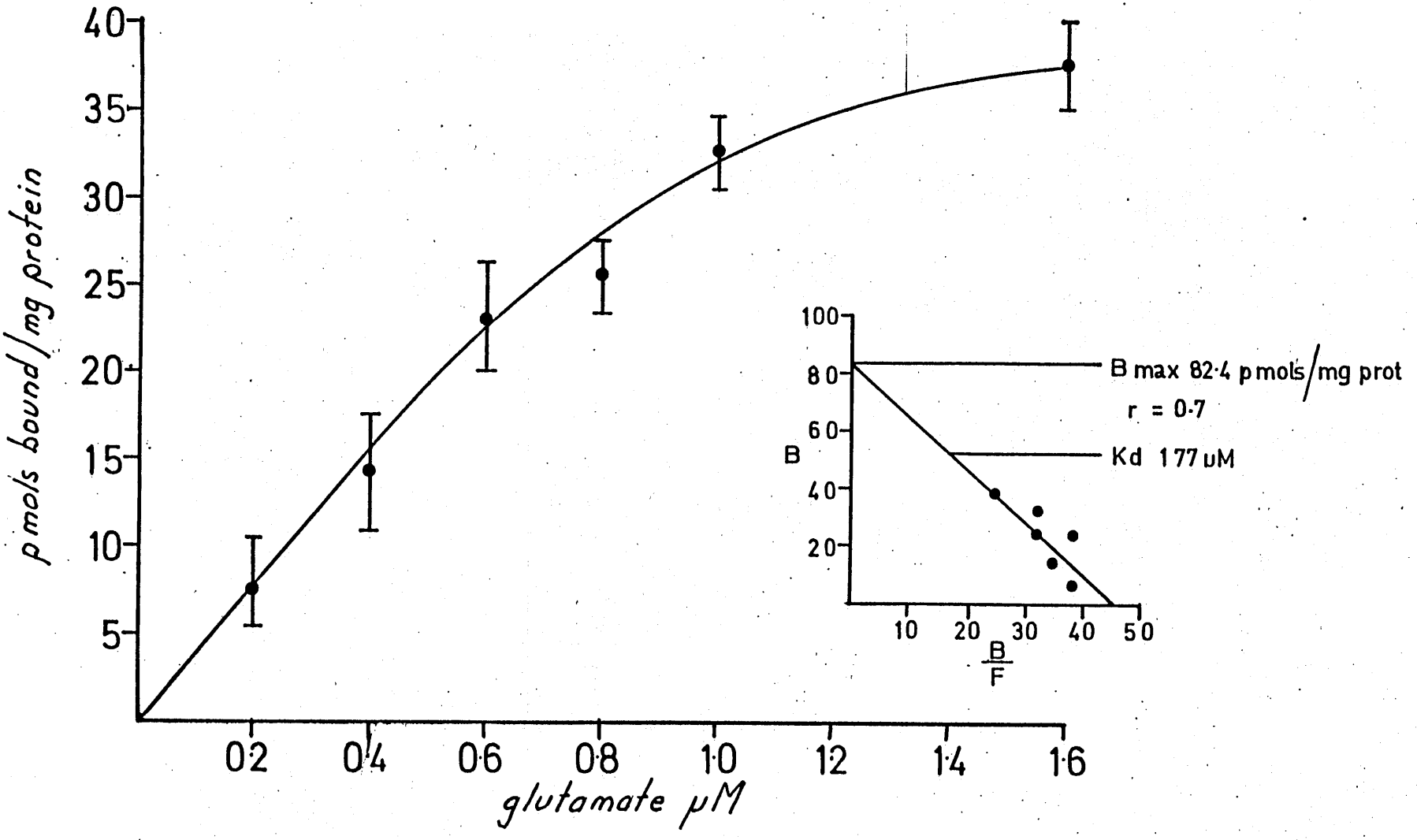
The ontogenetic profile of both sites shows the simultaneous development of Na^+ dependent and Na^+ independent binding at similar rates until 20 days of age. Na^+ dependent binding continues to rise, albeit at a lower rate until the 50 day level is reached. It is not surprising to find such a rapid rise in the number of sites during the first three weeks of life if what are being measured are parameters of a transmitter system. This is because, as already stated, although the number of neurons have reached their full complement by birth, the development of the dendritic tree and synaptogenesis are largely post-natal events in the cerebral cortex. As there is considerable evidence to show that excitatory post synaptic receptors are predominantly located on dendrites, a rapid expansion of dendritic arborization would be reflected in a simultaneous rapid increase in the total number of binding sites. The slower rise in the number of Na^+ independent binding sites up to 50 days could represent a population of late - developing synapses.

In contrast, Na^+ dependent binding shows a significant fall in the total number of binding sites from 20-30 days ($p < 0.01$). If these binding sites are subserving a reuptake function in glutamatergic transmission, then it may be that this fall could be due to a degeneration of presumptive uptake sites after commitment of others to functional

FIG. 56

The specific Na^+ dependent binding of ^{3}H-L-Glu to cortical membranes prepared from 20 day old rats, measured at a L-Glu concentration of 0.6 μM. Each point represents the mean $\pm$ SEM of 6 separate experiments. Kinetic parameters were estimated from a Scatchard plot of the data which is shown as an inset on the graph.

FIG 5b


40
35
30
25
20
15
10
5
pmols bound/mg protein
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
glutamate μM
100
80
60
40
20
B
10
20
30
40
50
B/F
B max 82·4 pmols/mg prot
r = 0·7
Kd 177 μM

glutamatergic pathways "hard wired" at the time of synaptogenesis. The degenerating uptake sites could thus represent a surplus to requirement and "make way" for uptake sites subserving other modes of neurotransmission. The subsequent sharp rise in levels of binding up to 50 days may be a consequence of the development of sites on rapidly proliferating glial cells surrounding glutamatergic synapses. There is considerable evidence to indicate that uptake by glial cells, rather than neuronal elements, is of prime importance to the termination of transmitter function (Balcar et al, 1977; Henn, 1976; Benjamin and Quastel, 1975). This is reflected in the large increase in site density from 82.4 p mols bound/mg protein at 20 days to 210 p mols bound/mg protein at 50 days, although the fact that the Kd values for Na^+ dependent binding are similar at both age points suggests that the same site is being measured in both cases.

A comparison of the kinetics of Na^+ independent binding at 20 days and 50 days shows rather unexpected results that are more difficult to explain. In contrast to reports on the development of other transmitter systems where the Kd remains the same throughout postnatal development, the Kd of Na^+ independent glutamate binding is twice the value (i.e. lower affinity) at 20 days than at 50 days. It may be that what is being measured at this stage is not a binding site solely for glutamate but an acidic amino acid receptor that can loosely bind either glutamate or aspartate. It may be only after efferent input and synapse formation that receptors become specific for either aspartergic or glutamateric transmission and will thus show higher affinity for one or the other of these agonists. Although post-synaptic receptor sites are present prior to synaptic innervation there

is evidence that complete maturation of these receptors only takes place after functional commitment of pre and postsynaptic cells to a particular mode of transmission (Changeux and Danchin, 1976). Although the results presented so far do not permit a more detailed argument in favour of this explanation of changing affinity constants throughout development, a discussion of this idea will be expanded upon in later sections.

5.3 Antagonism

The ability to demonstrate binding of glutamate to synaptic membranes that is saturable and of high affinity is not sufficient, in itself, to conclude the presence of specific post synaptic receptors. The case for doing so would, however, be considerably strengthened if it could be shown that the structural requirements forced on the ligand by the *in vitro* binding site were very similar, if not identical, to the physiological receptor. Thus, pharmacological agents that are either agonists capable of mimicking the physiological effect, or antagonists capable of blocking or reducing the effect of receptor activation should displace a ligand bound *in vitro* to such a site with the same order of potency displayed *in vivo*.

Since the initial studies of the excitatory action of glutamate and aspartate by Curtis (1965) and Curtis and Watkins (1963), other amino acids have been tested for their ability to evoke E.P.S.P.'s. Over 100 such compounds have been identified in this way (Watkins, 1978) and on the basis of a consideration of their structures, Curtis and Watkins (1960) suggested that a 3 point attachment site is the most likely configuration of the receptor activated by these amino acids. Of these 3 points, two will interact with anionic groups, one of which will be a carboxylate group and the 3rd will interact with a cationic

group which will (at physiological pH) be an associated amino group. The 2nd anionic group can vary considerably (hence the large number of excitatory agonists). Structural alterations which abolish ionizability of any of these groups drastically diminishes excitatory effects.

Among the most potent of these excitatory amino acids are kainic acid, quisqualic acid (being approx. 50 times more potent than glutamate) and N-methyl D-aspartic acid, which is around 20 times more potent than glutamate (Watkins, 1978). The crucial questions in terms of increasing our understanding of the mechanisms used in the CNS to mediate transmission must be; are the excitatory effects brought about by interaction with a single class of receptors or are there unique populations of receptors specifically activated by one or more of the very few of these amino acids that naturally occur in the CNS?

Glutamate, and to some extent aspartate, are very flexible molecules. Glutamate can assume configurations ranging from a fully extended form, where the separation of the two anionic carboxyl groups is at a maximum of approx. 0.46 nM, or a fully folded form where they are at a minimum of 0.18 nM from each other. The maximal separation of the carboxyl groups in aspartate is 0.38 nM and the minimum is 0.25 nM. From such considerations the possibility arises that at least 3 subsets of physiologically relevant amino acid receptors may be present on neuronal cell membranes in the mammalian CNS. One may demand maximal separation of COO^- groups and would therefore be glutamate preferring, aspartate being unable to extend to make the 3 point interaction. Another may accept glutamate in its fully folded form and a 3rd would be aspartate preferring, but would accept glutamate were it to assume an intermediate, partially extended configuration.

Kainic acid (KA) is a glutamate analogue that is fixed into a position where the COO^- groups are maximally separated by the conformational restrictions imposed by a pyrollidine ring. Thus KA was originally proposed as a preferential agonist for the extended glutamate receptor (Johnson, 1974).

Quisqualate, (Quis), unlike the other potent amino acid agonists KA and N-methyl-D-Aspartate (NMDA), possesses only one carboxyl group, but has a secondary amino group adjacent to two carbonyl groups in an unusual oxadiazolidinedione ring system. The two carbonyl groups act as a strong influence, reducing the electron density over the N-atom of the amine group leaving the hydrogen atom only weakly associated so that it acts as a functional acidic hydrogen with a pka of 4.15 (Takemoto, 1976), very similar to the side chain carboxyl group of glutamate (pka 4.32). Loss of this proton at physiological pH could result in a redistribution of the anionic charge throughout the ring, resulting in the oxygen atom on C3 becoming anionic. The anionic carbonyl in Quis is at the same distance from the primary amino group as the COOH in glutamate from its own amino group. Quis can thus be considered, on these structural considerations, as a glutamate analogue which, although retaining some degree of flexibility, sterically prefers a folded configuration and may have preferential affinity for the fully folded glutamate receptor.

N-methyl-D-Aspartate (NMDA) is an analogue of aspartate where the primary amino group has become a secondary amino group by the substitution of a methyl group. Although most compounds carrying substitutions in the amino group are considerably weaker as excitatory agonists than their parent compound, this is not true of NMDA. Because of its striking similarity to aspartate, NMDA may well act at postsynaptic receptors that are aspartate preferring but which may be capable of accepting glutamate

in a partially folded (or partially extended!) form.

McLennan et al, as early as 1968, had shown a lack of parallelism in the responsiveness of thalamic neurons in cat to L-glu and NMDA, and proposed at that time at least two different populations of receptors. Duggan (1974) suggested that L-Glu and L-Asp may act on different receptors on the basis of the differential sensitivity of different groups of cat spinal neurons to the two agonists. McCulloch et al, also in 1974, confirmed these findings and demonstrated that the relative potencies of KA and NMDA parallelled the relative potencies of L-Glu and L-Asp respectively. This is certainly in keeping with the theoretical considerations and in addition it is well established that in invertebrates several different classes of receptor, both excitatory and inhibitory, do exist (Cull Candy, 1976).

More evidence in favour of this discrimination between different classes of amino acid receptors came from studies of the effects of divalent metal ions on the depolarizing responses to amino acid agonists in the frog spinal cord. Evans et al (1977) showed that responses to NMDA were considerably depressed by low concentrations (threshold 10 μM) of Mg^{++}, Ca^{++} and Ni^{++}, whilst responses to KA, L-Glu and Quis were unaffected. Significantly, responses to L-Asp were more sensitive to Mg^{++} than were responses to L-Glu, confirming the belief that NMDA may be acting preferentially at "aspartate preferring" receptors. Similar results were obtained at around the same time using cat spinal cord as the test system.

Until this time, investigations of amino acid receptors were made using agonist properties only and positive discrimination between different types of receptor proved difficult due to the marked degree of overlap of agonist effects on different groups of neurons. The studies with Mg^{++}

at least suggested that differentiation of receptor types may be possible by exploiting agents that antagonise excitatory amino acid responses and a range of organic compounds, both naturally occurring and synthetic amino acid analogues, were screened for specific antagonistic properties. Continuing exhaustive studies of structure activity relations of amino acid excitants, mainly by Watkins and co-workers in Bristol, but also by McLennan and co-workers in Canada and by Curtis and co-workers in Australia have subsequently led to developments in this area. Such studies have resulted in the recent discovery of selective antagonists that seem able to distinguish, not only between excitation produced by amino acid compared to non amino acid agonists, but also between the agonist action of different amino acids.

Of these antagonists, two proved initially most useful as tools for the investigation of receptor differences for the excitant amino acids. One of these was the diethylester of glutamate (G.D.E.E.) and the other is one of a class of antagonists including longer chain mono - and diaminocarboxylic acids of which the best characterized is -D∝ Aminoadipic acid (D∝AA) (Biscoe et al, 1978; Evans et al, 1978; Hicks et al 1978; Lodge et al, 1978; McLennan and Hall, 1978).

GDEE was originally reported to have a selective effect in abolishing excitations induced by L-Glu when contrasted with those produced by L-Asp (Haldeman et al, 1972; Haldeman and McLennan, 1972). In addition subsequent studies have shown that iontophoretically applied GDEE blocks Quis induced, but not KA induced, responses of cat spinal neurons (McLennan and Lodge, 1979; Davies and Watkins, 1979). Such initial studies strongly supported the view that GDEE may be selective for glutamate receptors, accepting glutamate in a folded configuration.

In contrast D∝AA proved ineffective in reducing glutamate induced excitations, but instead antagonised potently responses to L-Asp, and more particularly to NMDA. Investigation of the antagonist effects of D∝AA and GDEE on several agonist responses show almost reciprocal effects as summarized in Table 52 (Taken from McLennan, 1981). McLennan has also suggested that substances appearing near the middle of each column, like homocysteate, D-Glu and L-Asp may be able to interact, to some extent, with both classes of receptor.

The position with respect to D-Glu is particularly interesting because,although it is a neuronal excitant with about half the potency of L-Glu,its action is preferentially blocked by D∝AA, unlike the action of L-Glu. L - and D-Glu can only "fit" the same receptor if they (and therefore the receptor?) are in a relatively extended configuration (McLennan, 1981). D-Glu cannot fit a fully folded L-Glu receptor and may therefore act as a L-glu antagonist at such a receptor by causing steric hindrance preventing the access of L-Glu although it cannot itself form an active complex with the receptor. The excitatory action of D-Glu then, may be mediated by its interaction with the partially extended, D∝AA sensitive, glutamate receptor. These theoretical considerations have been confirmed by the demonstration that the action of D-Glu on D∝ AA treated neurons is to antagonise the remaining response to L-Glu,although it does not do so in the absence of D∝AA when it acts itself as a neuronal excitant (McLennan 1981).

Thus in summary, pharmacological studies of excitant amino acid receptors have indicated the possible presence of 3 subsets of receptors:-

1. Preferentially activated by NMDA, L-Asp, D-Glu and L-Glu in a partially extended form (and in that order of potency) and antagonized by D∝AA.

TABLE 5.2 Antagonism of amino acid-induced excitations

by GDEE	by DαAA
L-glutamate	N-methyl-D-aspartate
Quisqualate	N-methyl-L-aspartate
D-aspartate	Ibo tenate
L-aspartate	ADCP
D-glutamate	L-homocysteate
D-homocysteate	D-homocysteate
L-homocysteate	D-glutamate
Ibotenate	L-aspartate
ADCP	D-aspartate
N-methyl-L-aspartate	Quisqualate
N-methyl-D-aspartate	L-glutamate

2. Preferentially activated by L-Glu in its folded form, and by Quis, and antagonized by G.D.E.E. (and D-Glu).
3. Preferentially activated by KA and possibly by Glutamate in a fully extended form.

On the basis of the above considerations the ability of D-Glu L-Asp, KA, NMDA, D AA, and L-Glu to displace ^{3}H-L-Glu was investigated. The displacers were added to the assay tubes in concentrations varying between 10^{-9} and 10^{-4}M together with ^{3}H-L-Glu at a concentration of 0.6 M. 100% specific binding was the amount by which the binding of ^{3}H-L-Glu (0.6 M) was reduced in the presence of 0.1 mM L-Glu compared to tubes containing only the radioligand. The displacing ability of the above named compounds was studied for L-Glu bound to both the Na^{+} dependent and the Na^{+} independent sites at both the 50 and the 20 day age points.

Fig. 57 shows the structures of the compounds utilized in the displacement studies together with Quis which was not available commercially at the time.

RESULTS

Table 5.3 shows the IC50 values of the amino acids acting as displacers of specific ^{3}H-Glutamate (0.6 M) binding to membranes prepared from rat cerebral cortex at 20 days and 50 days of age. IC50 values were determined from log dose/percent inhibition plots, shown in Figs. 58 (50 days) and 5.9 (20 days). Considering first the displacement of L-Glu bound to the putative postsynaptic receptor site (i.e. the Na^{+} independent site) in adult rats, it can be seen that although L-Asp can displace L-Glu bound to this site it does so with a potency 10 times weaker than L-Glu itself. The comparable IC50 values are 6.4 M for L-Asp and 0.65 M for L-Glu. None of the other compounds, neither agonists nor antagonists, were effective in displacing glutamate from this site (all exhibiting considerably less than 50% inhibition at concentrations of 0.1 mM).

FIG. 57

The structures of the amino acid agonists and antagonists used in the displacement studies reported in this thesis as probes for the structural requirements demanded by the L-Glu and L-Asp binding sites on rat cortical membranes.

Asp = L - Aspartate.

NMDA = N-methyl-D-Aspartate.

Glu = L-Glutamate.

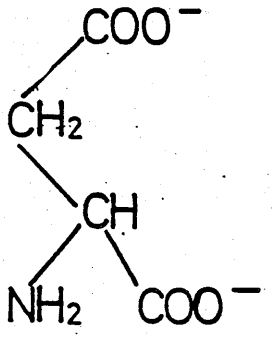
KA = Kainate.

Quis = Quisqualate.

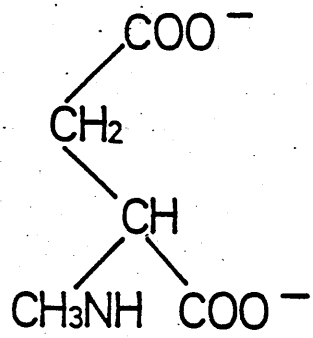
D&AA = D ∝ Amino-adipate.

FIG 57

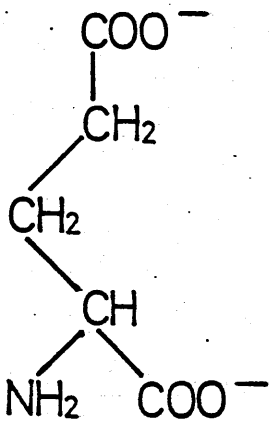
ASP



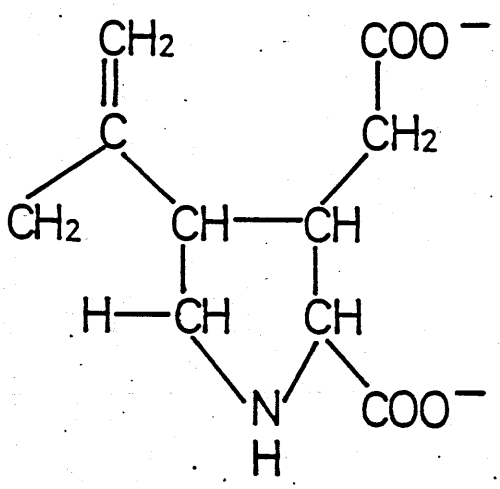
NMDA



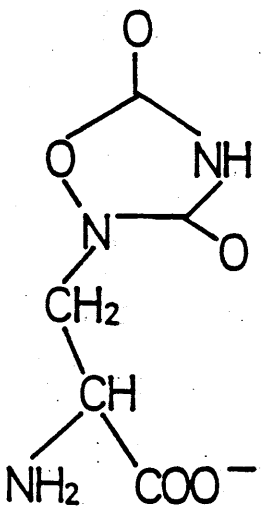
GLU



KA



QUIS



D∝AA

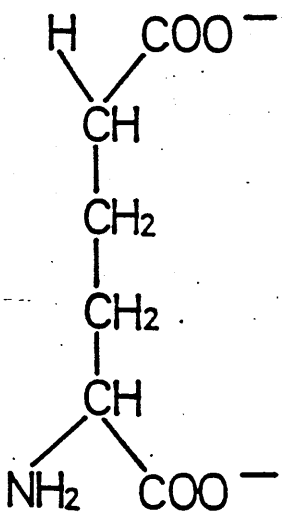


FIG. 58

The Inhibition of binding of ^{3}H-L-Glutamate by excitant amino acids to membranes prepared from the cortex of 50 day old rats.

A - Inhibition of Na^{+} independent binding.

B - Inhibition of Na^{+} dependent binding.

■ - L - Glutamate.

□ - L - Aspartate.

○ - D - Aspartate.

All other analogues displaced considerably less than 50% of the binding at the highest concentration tested (10^{-4}M). Each point is the mean of at least 4 separate experiments which did not differ by more than 5%.

The displacement curves in (A) are essentially parallel to each other although there is an order of magnitude difference between the displacement abilities of the two ligands.

In B the displacement curves for L-Glu and L-Asp are very similar and they exert their effect over the same order of magnitude indicating that they are probably interacting with the same site.

The results of all displacement studies with ^{3}H-L-Glutamate are summarised in Table 5.3.

FIG 5B

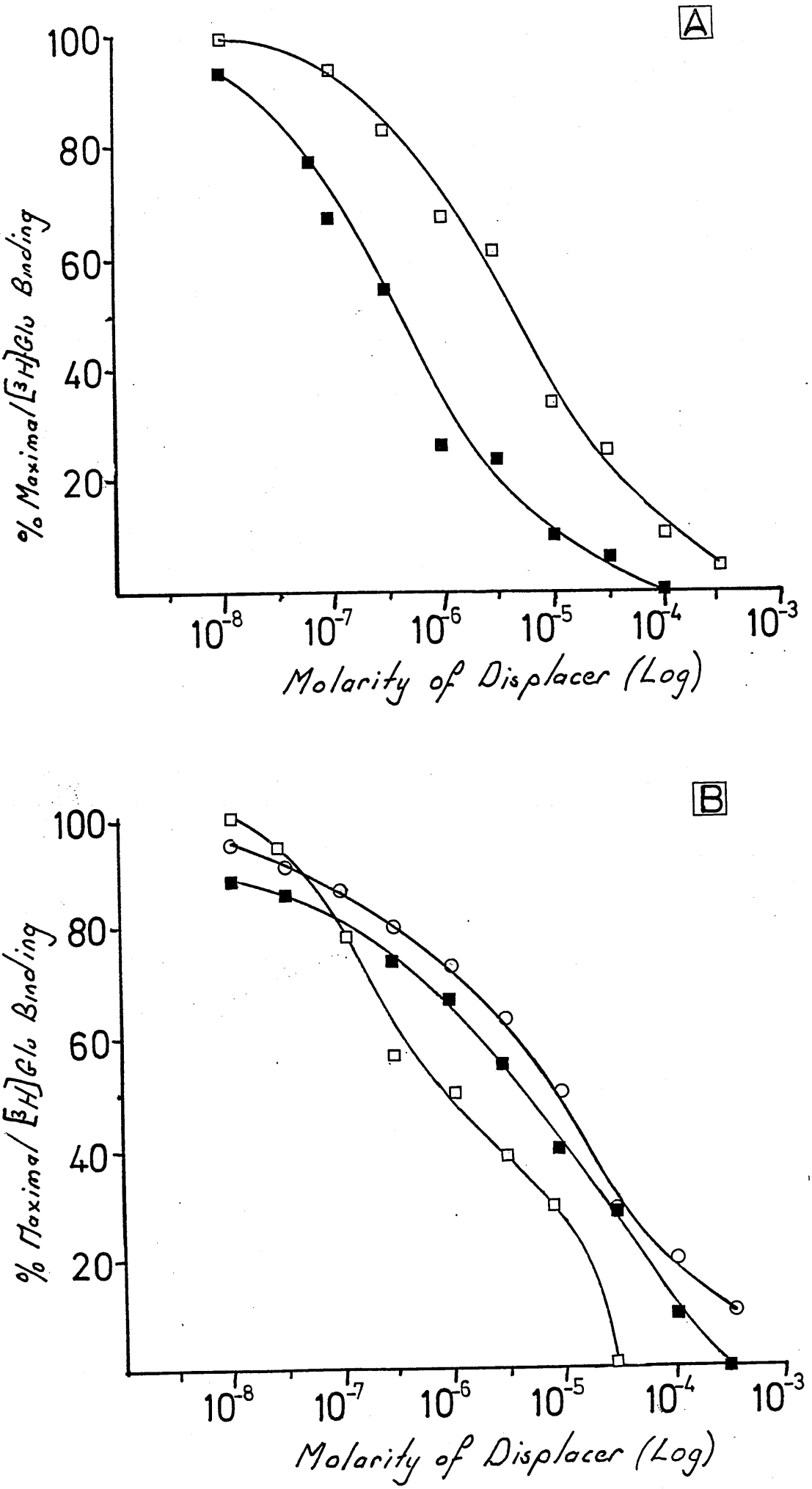

A
100
80
60
40
20
% Maximal [3H]Glu Binding
10-8
10-7
10-6
10-5
10-4
10-3
Molarity of Displacer (Log)
B
100
80
60
40
20
% Maximal [3H]Glu Binding
10-8
10-7
10-6
10-5
10-4
10-3
Molarity of Displacer (Log)

Fig. 59

Inhibition of the binding of ^{3}H-L-Glutamate by excitant amino acids to membranes prepared from the cortices of 20 day old rats.

A - Inhibition of Na^{+} independent binding.

B - Inhibition of Na^{+} dependent binding.

■ - L Glutamate.

□ - L - Aspartate.

● - D ∝ Amino Adipate.

△ - N-methyl - D - Aspartate.

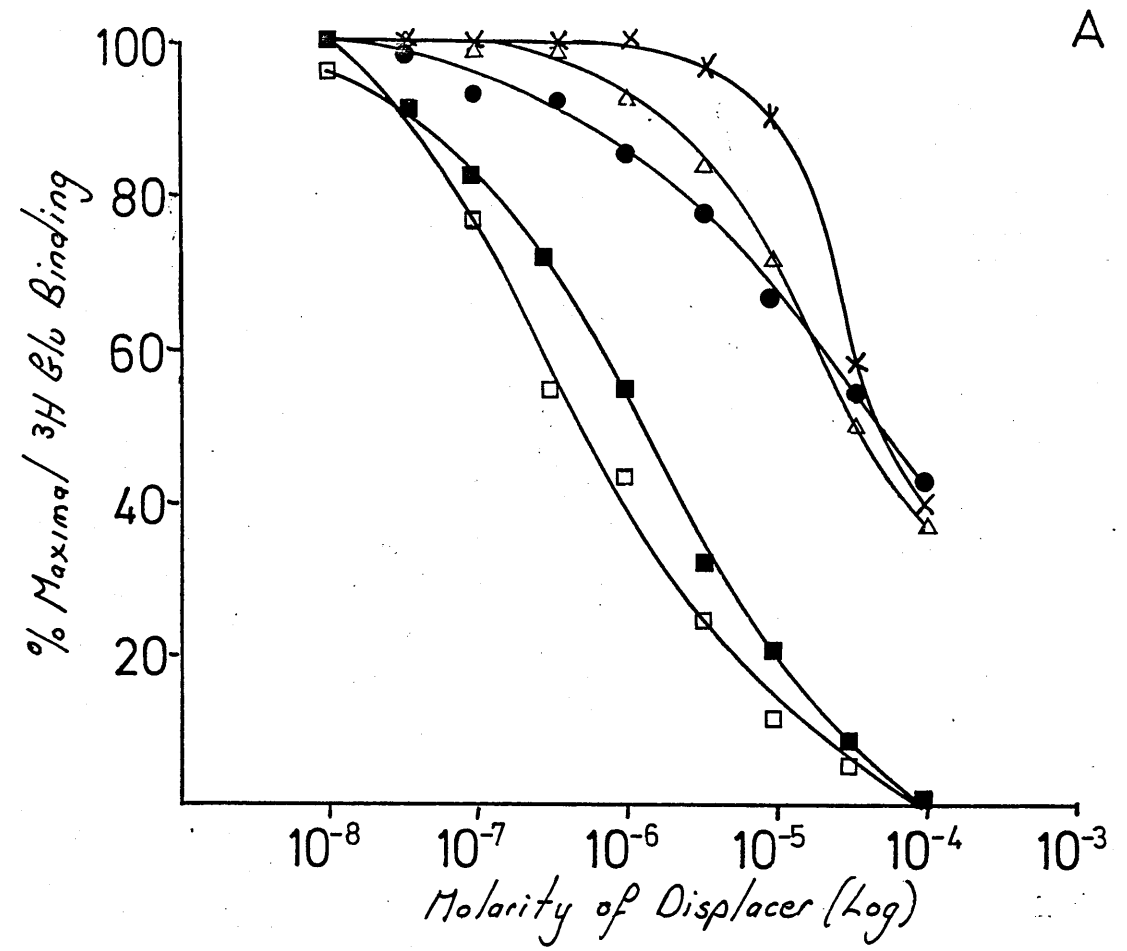
× - Glutamate diethyl ester.

Kainate and D—Glutamate were ineffective as displacers of Na independent binding at 10^{-4}M. All other analogues not shown on B were ineffective at displacing Na^{+} dependent binding at a concentration of 10^{-4}M. See text for discussion.

Each point is the mean of 4 separate determinations which did not differ by more than 10%.

The results of all displacement studies with ^{3}H-L-Glutamate are summarised in Table 5.3.

FIG 5g


A
100
80
60
40
20
% Maximal / 3H Glu Binding
10-8
10-7
10-6
10-5
10-4
10-3
Molarity of Displacer (Log)

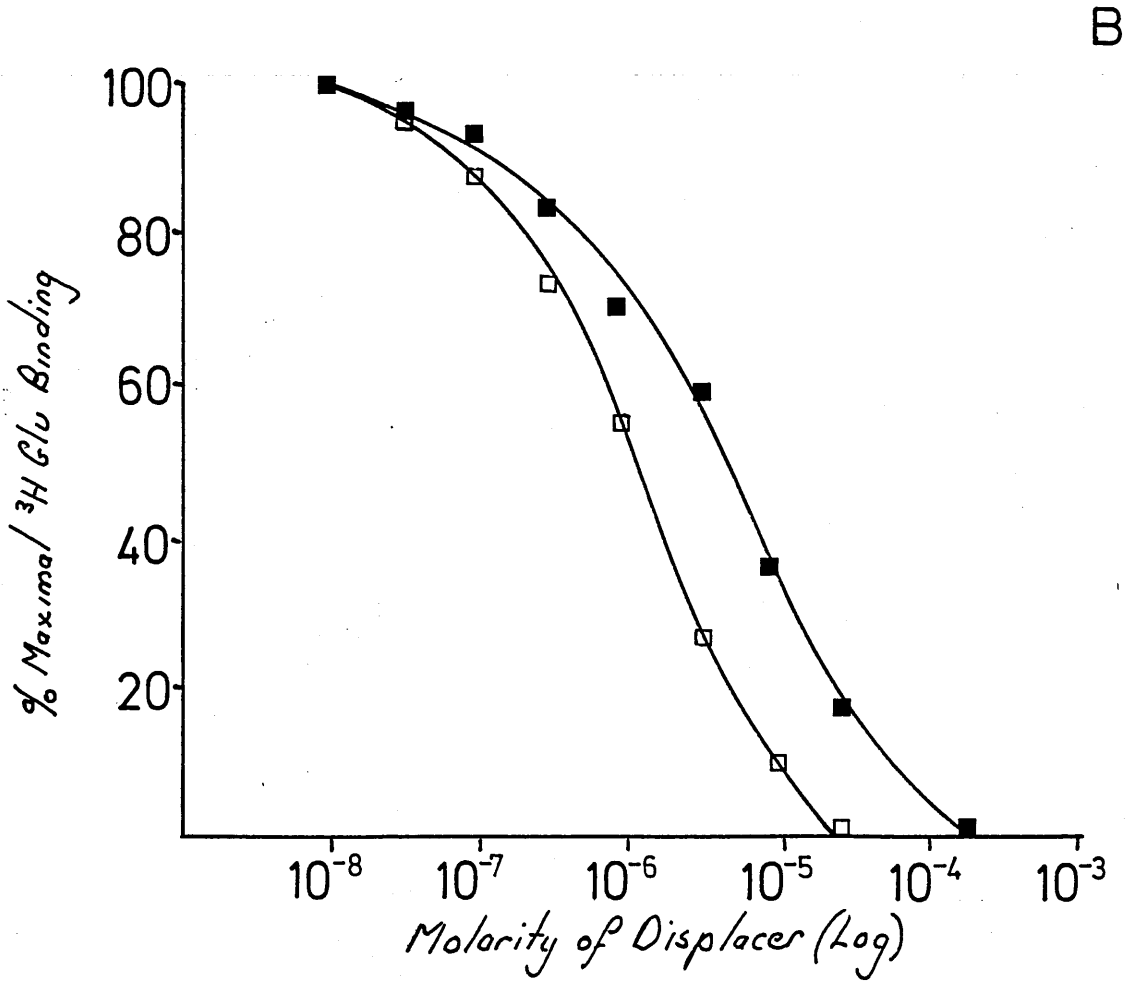

B
100
80
60
40
20
% Maximal / 3H Glu Binding
10-8
10-7
10-6
10-5
10-4
10-3
Molarity of Displacer (Log)

TABLE 5.3 IC_{50} values of a number of amino acid analogues (concentration range 10^{-9} - 10^{-4} M) acting as displacers of (^{3}H) glutamate (0.6 µM) specifically bound to membranes prepared from the rat cerebral cortex at 20 and 50 days of age.

	20 DAY		50 DAY	
	Na^+ dependent	Na^+ independent	Na^+ dependent	Na^+ independent
L-glutamate	6.4 µM 6.4×10^{-6} M	1.6 µM 1.6×10^{-6} M	6.5 µM 6.5×10^{-6}	.65 µM 6.5×10^{-7}
D-glutamte	$> 10^{-4}$	$> 10^{-4}$	1.0 µM 1.0×10^{-6}	$> 10^{-4}$
L-aspartate	3.2 µM 3.2×10^{-6} M	0.78 µM 7.8×10^{-7}	8.6 µM 8.6×10^{-6}	6.4 µM 6.4×10^{-6}
Kainate	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$
glutamate diethyl ester	$> 10^{-4}$ M	10^{-4} - 10^{-5} M	$> 10^{-4}$	$> 10^{-4}$
D ∝ amino adipate	$> 10^{-4}$ M	10 µM $> 10^{-5}$ M	$> 10^{-4}$	$> 10^{-4}$
N methyl D aspartate	$> 10^{-4}$ M	10 µM $> 10^{-5}$ M	$> 10^{-4}$	$> 10^{-4}$

In contrast L-Asp displaces L-Glu bound to this same site in 20 day old animals with a potency twice as high as that of L-Glu itself. The respective IC50 values in this case are 0.78μm for L-Asp and 1.6μM for L-Glu. Especially interesting is the observation that L-Glu can also be displaced from its Na^+ independent binding site at 20 days by the agonist and L-Asp analogue NMDA, as well as by the antagonists GDEE and DL∝AA with IC50 values around 10μM. To reiterate, all 3 compounds are ineffective at displacing L-Glu at 50 days of age.

The pharmacological specificity of the Na^+ dependent site changes markedly during the period between 20-50 days only in respect of its ability to recognise D-Glu . This biologically inactive stereoisomer of glutamate will not displace L-Glu bound to its Na^+ dependent site (putative re-uptake site) at 20 days, but will do so at 50 days with an IC50 value of 1.0 μM, some 6 times higher than the IC50 value of L-Glu itself. L-Asp shows a slightly higher ability to displace L-Glu from its Na^+ dependent site at 20 days than it does at 50 days. The IC50 value of glutamate itself is greater at 50 days than at 20 days (the respective IC50 values are 6.5μM and 1.6μM) but neither the two agonists NMDA or KA or the two antagonists GDEE and DL∝AA have any affinity for this binding site at either of the two age points. Interestingly KA alone of the test compounds will not displace L-Glutamate bound either in the presence or absence of Na^+ at either age.

Discussion

A consideration of the result that L-Glu is much more effective at displacing ^{3}H-L-Glu bound to the Na^+ independent site at 50 days than is L-Asp implies that the L-Glu binding site is specialized to accept L-Glu either in its fully extended (maximal separation of COOH groups) or

fully folded (minimal separation of COOH groups) forms. The adoption of an intermediate configuration by the receptor would make it accessible to both L-Asp and L-Glu.

This view is reinforced by the fact that neither of the aspartate analogues NMDA and DαAA displace glutamate. As these two substances have been shown to have very specific agonist and antagonist activity respectively on those neurons that are aspartate preferring (see e.g. Watkins, 1978; Johnson, 1978), glutamate would seem to be acting here at a different receptor or receptors than those sensitive primarily to NMDA or DαAA.

The glutamate antagonist GDEE has been proposed as an agent specifically antagonising those responses produced by L-Glu and by the conformationally restricted folded glutamate analogue, Quisqualate (McLennan, 1981). So it may be expected that if L-Glu is interacting with the reported Na^+ independent binding site on cortical membranes in a folded form it would be displaced by GDEE. This was not the case in this present study and may possibly be explained by the fact that most of the work characterising the effect of this antagonist has been carried out by looking at its effect on induced responses in the spinal cord where the structural requirements of the receptor may be different than in the cortex. It has been subsequently reported that there may be distinct regional differences in the effect of this antagonist in terms of both its potency and selectivity (Clarke and Straughn, 1977; McLennan and Wheal, 1976). Although GDEE seems to antagonise response to L-Glu in most situations, responses induced by L-Asp also seem susceptible to GDEE antagonism, albeit to a lesser extent (see e.g. Johnson, 1978). This could imply that G.D.E.E. may in fact be acting to displace glutamate bound only to aspartate preferring sites, although its lack of effect on

NMDA responses would seem to preclude this explanation. Nevertheless, there is some evidence from studies with rat spinal cord that in this tissue at least, sites of activity of L-Glu and G.D.E.E. may be different. Nistri and Constanti (1975) and Bailey and Phillis (1976) have shown in these studies that GDEE did not affect glutamate induced depolarization, but exhibited a depolarizing action of its own, suggesting an agonist affect at a separate site from that mediating glutamate responses.

Other observations that G.D.E.E. can influence the spontaneous activity of neurons (Bailey et al, 1976; Altman, et al 1976) may point to the fact that its actions may be relatively non-specific so that quite large doses may be necessary to demonstrate a displacement of any specifically bound ligand. This suggestion is upheld by the observation that depression of visually induced depolarization in the Lateral Geniculate Nucleus by GDEE is only achieved by doses of the antagonist that are sufficiently large to cause non-specific depression of chemically induced responses (Kemp and Sillito, 1979).

Because of the reported regional variations in GDEE effects it is obviously significant that excitations produced by the pyramidal tract projection into the cerebral cortex have been shown to be antagonized by GDEE (Stone, 1976).

Comparable <u>in vitro</u> studies of the displacement of binding from its Na^+ independent site show differing effects in relation to the activity of GDEE. Michaelis et al,(1976)were among the first to report binding of L-Glu to brain membranes and reported that GDEE was a very poor displacer of bound ^{3}H-L-Glu. Roberts, in the same year, reported binding of ^{3}H-L-Glu to synaptic membranes prepared from the cerebral cortex of rats that could in contrast be inhibited by GDEE by 60%. De Plazas and DeRobertis (1976)

succeeded in extracting and isolating a proteolipid fraction from synaptic membranes prepared from the cerebral cortex of rats that had binding sites for L-Glu, L-Asp and the inhibitory amino acid GABA. Discrimination between the glutamate and aspartate sites was possible because GDEE did in fact inhibit the Na^+ independent binding of L-Glu by 65%, whilst the binding of L-Asp was only inhibited 24%.

Roberts and Sharif (1981) have recently reported that GDEE is ineffective as a displacer of ^{3}H-L-Glu bound to cerebellar membranes, whilst Biziere et al (1980) confirmed the earlier result of Michaelis et al (1974) that GDEE was relatively ineffective as a displacer of L-Glu bound to membranes prepared from whole brain.

Thus the relationship of GDEE to glutamate receptors is still very poorly characterized and accumulated evidence points to the use of caution in interpreting effects of GDEE on neurons <u>in vivo</u> or <u>in vitro</u>, (Johnson 1978). It is possible that further studies with GDEE may be more fruitful if carried out in conjunction with several very recently discovered antagonists that may prove more selective in their action and their relative potencies compared. The effects of these new antagonists will be discussed in later sections summarising the possible mode of interaction of glutamate with brain membranes.

If glutamate is not acting as its receptor in the folded (supposedly GDEE sensitive) format, it could therefore be interacting with a glutamate receptor accepting its ligand in an extended conformation. K.A. has been proposed as a specific ligand for such a site but was ineffective in displacing ^{3}H-L-Glu in these studies.

Since it was first proposed as a specific agonist for glutamate preferring receptors, considerable neuropharmacological evidence has accumulated to indicate that KA binding sites may not in fact be synonymous with those for glutamate (Hall et al, 1978). In keeping with this, Michaelis et al (1980) have succeeded in separating binding sites for the two agonists. KA also has differential effects to glutamate in stimulating brain cyclic nucleotides (Schmidt et al, 1975) and on ion distribution and ATP levels in striatal slices (Bizière and Coyle, 1978). Thus KA binding sites may represent either a very small population of glutamate binding sites that may in fact be extrajunctional or presynaptic, or, as suggested by Roberts (1981), KA may be binding to receptors for an as yet unidentified endogenous substance.

The failure of D-Glu to displace specifically bound L-Glu is surprising in view of its very similar excitatory potency. This agonist action of D-Glu, as previously mentioned, is probably mediated by an interaction with the aspartate preferring NMDA receptor (DαAA sensitive) so this result may indeed suggest that glutamate is combining with its receptor in its folded or extended form. D-Glu may be a weak antagonist at the extended Glutamate receptor but may not be able to displace L-Glu specifically bound, at the concentration used.

The fact that L-Asp and D-Glu are capable of displacing L-Glu bound to the Na^+ dependent site in adults is quite consistent with the observation that these three substances share the same Na^+ dependent transport process, as previously discussed.

The structural requirements of the L-Glu Na^+ independent binding site are different at 20 days than at 50 days. This is especially evident when

the ability of L-Asp to displace the binding is compared at the two age points. L-Asp displaces L-Glu at 20 days with a potency twice that of L-Glu and 20 times higher than its ability to do so at 50 days. In addition, the agonist NMDA and the antagonists GDEE and DαAA all displace the binding at 20 days with an IC50 of about 10μM. The fact that the binding site at this age is capable of accepting both aspartate-like and glutamate - like analogues points to a considerable flexibility in the conformation of the receptor molecule that is not present at 50 days. The receptor at this stage may exist in a form that is relatively mobile within the lipid environment of the membrane, due to a lack of sufficient hydrophobic interactions at its surface. This would allow the acceptor site of the receptor to assume slight changes in configuration so that the charged groups with which the different ligands interact can shift their position relative to each other depending on which of the ligands bind.

The shift to a conformation only capable of accepting glutamate, could be the result of increased activity of the presynaptic cell preferentially utilizing one or the other of the amino acid transmitters, in this case presumably glutamate. This in turn may initiate a chain of events in the postsynaptic cell that could lead to the covalent modification of the receptor protein. This modification may result in the exposition of more hydrophobic residues capable of interacting with the surrounding hydrophobic (lipid) environment thus 'crystallizing' its local environment and stabilising the receptor in one configuration - in this case the glutamate preferring configuration. This speculative interpretation of the data is in keeping both with the mobile receptor-model for agonist action and also with the mechanism of selective stabilization of neuronal connections by functional verification, as proposed by Changeux and Danchin (1976). The 20 day age point in the rat corresponds to the time at which maximum rates of synaptogenesis are

occurring and it is quite conceivable that presumptive synapses formed at this stage may later become utilized by a particular pathway and manifest themselves as subservient to a particular mode of transmission later in development.

In summary, the data reported on the effects of amino acid agonists and antagonists on the binding of ^{3}H-L-Glu to rat cortical membranes probably indicate that glutamate is binding to a class of receptors that is glutamate preferring as opposed to aspartate preferring. It was not, possible, however, to discriminate between glutamate receptors accepting L-glu in its extended form or in its folded form. The glutamate antagonist GDEE, which has been proposed to specifically antagonise responses via the extended form of the receptor, was ineffective in adult rats although, together with the aspartate analogues NMDA and DαAA, it did displace L-Glu bound to membranes from 20 day old rats. These results were mainly discussed in terms of the development of a binding site for acidic amino acids that fulfills many of the neurochemical criteria for a postsynaptic transmitter receptor. Although relatively non-specific in terms of its discrimination between different amino acids at an early age, this receptor becomes specified for glutamatergic transmission later in development as shown by the changing affinity of this site to glutamate and aspartate analogues.

The agonist kainic acid, proposed originally as specific for the extended glutamate receptor, was ineffective at displacing any bound glutamate at either age. Interest in this molecule mainly arose because it is a powerful neurotoxin, as well as being a potent neuronal excitant and glutamate analogue. Its neurotoxic activity is most pronounced after injection into the striatum where it destroys neuronal cell bodies whilst sparing axons passing through or terminating in that region. The

neuroanatomical effects of KA neurotoxicity very closely resemble the pathology of Huntingtons Chorea. Because of its similarity to glutamate, the possibility arose that this disease may be related to defects in the pathways mediating glutamatergic transmission. Many of the initial reports of the effects of KA strongly supported its candidature as a specific agonist for the glutamate receptor. This culminated in a report of the in vitro vinding of KA to brain membranes (Simon et al, 1976) and provided the initial stimulus and starting point for work on the problem of glutamatergic transmission as reported in the next section.

5.4 Kainic Acid (KA)

One of the least effective of the amino acid analogues tested for their ability to displace either glutamate or aspartate from their binding sites was kainic acid. This naturally occurring amino acid was first isolated from the seaweed Digenea Simplex in 1953 by Takemoto. This seaweed (a red algae of the class Rhodomelaceae) had been used therapeutically for centuries as an antiascaris agent and Takemoto was able to demonstrate that the KA isolated from the seaweed retained these powerful antihelmintic properties and was indeed the active ingredient. There was evidence that KA acted by paralyzing ascarids and there were reports of a strong emetic action in dogs. The similarity of its chemical properties and structure to glutamate became of interest when neurophysiological studies demonstrated the potent excitatory properties of glutamate both at he invertebrate NMJ (Takeuchi and Takeuchi, 1964; Usherwood, 1969) and at neurons in the mammalian C.N.S. (Curtis and Watkins, 1963; Crawford and Curtus, 1964).

Initial studies demonstrated not only that KA was an extremely powerful neuronal excitant but that it also potentiated the action of glutamate on mammalian CNS neuronal cell membranes. The relationship between the possible mechanism of action of glutamate and KA was further strengthened when it was discovered that, like glutamate, KA was a potent neurotoxin, some 300 x more powerful than glutamate itself, paralleling its greater effectiveness as a neuronal excitant (Olney, et al 1974). Kainic Acid causes lesions characterized by an acute degeneration and loss of neuronal perikarya and their dendrites, whilst axons either termination in the area, or passing through, are spared. In

addition these cytopathological and neurochemical changes associated with KA neurotoxicity in the striatum were strikingly similar to those found in patients with the neuro-degenerative disease, Huntingtons chorea. This observation strongly implicated some fault in the glutamatergic system as a major component of the disease, given the very strong evidence that the afferent cortico-striatal pathway does indeed utilize glutamate as its transmitter (McGeer et al, 1977). Thus KA could be utilized as a tool to investigate the contribution of particular compartments to the neurochemical properties of specific brain regions (McGeer et al, 1978).

The similarities to glutamate so far reported were strongly suggestive of the possibility that K.A. may be exerting its neuroexcitatory and neurotoxic effects via membrane components that in natural conditions respond only to the acidic amino acids. If this was so, then KA may prove a much more discriminatory ligand (given its greater potency) for probing the localization and characteristics of glutamate binding sites in the CNS than glutamate itself. In addition, the conformational restrictions imposed on its structure by the presence of its pyrollidine ring means that it essentially corresponds to glutamate in an extended conformation. It would therefore be unlikely to interact with binding sites for the smaller molecule aspartate (Johnston et al, 1974; McCulloch et al, 1974) although glutamate may be able to do so in a folded configuration.

From these considerations the characteristics of the binding of ^{3}H-kainic acid to brain membranes was investigated by Simon et al (1975). They reported that the binding exhibited many properties consistent with the binding to a postsynaptic receptor accepting glutamate under physiological conditions. In particular, binding was saturable, of high affinity (kd 59 nM) with a low concentration of binding sites

(βmax; 1.03 pmols/mg protein) that were restricted to nervous tissue and were differentially located in various brain regions. Also, displacement studies did indeed suggest that binding may be to a population of amino acid receptors highly specific for glutamate as opposed to other excitatory amino acids.

On this basis kainic acid was implicated as an important pharmacological tool for the characterisation of endogenous glutamate receptors. The work reported in this thesis was initiated by a study of the binding of ^{3}H Kainic acid to membranes prepared from adult rat cortex. Tissue preparation and assay conditions were exactly as reported by Simon et al, (1975).

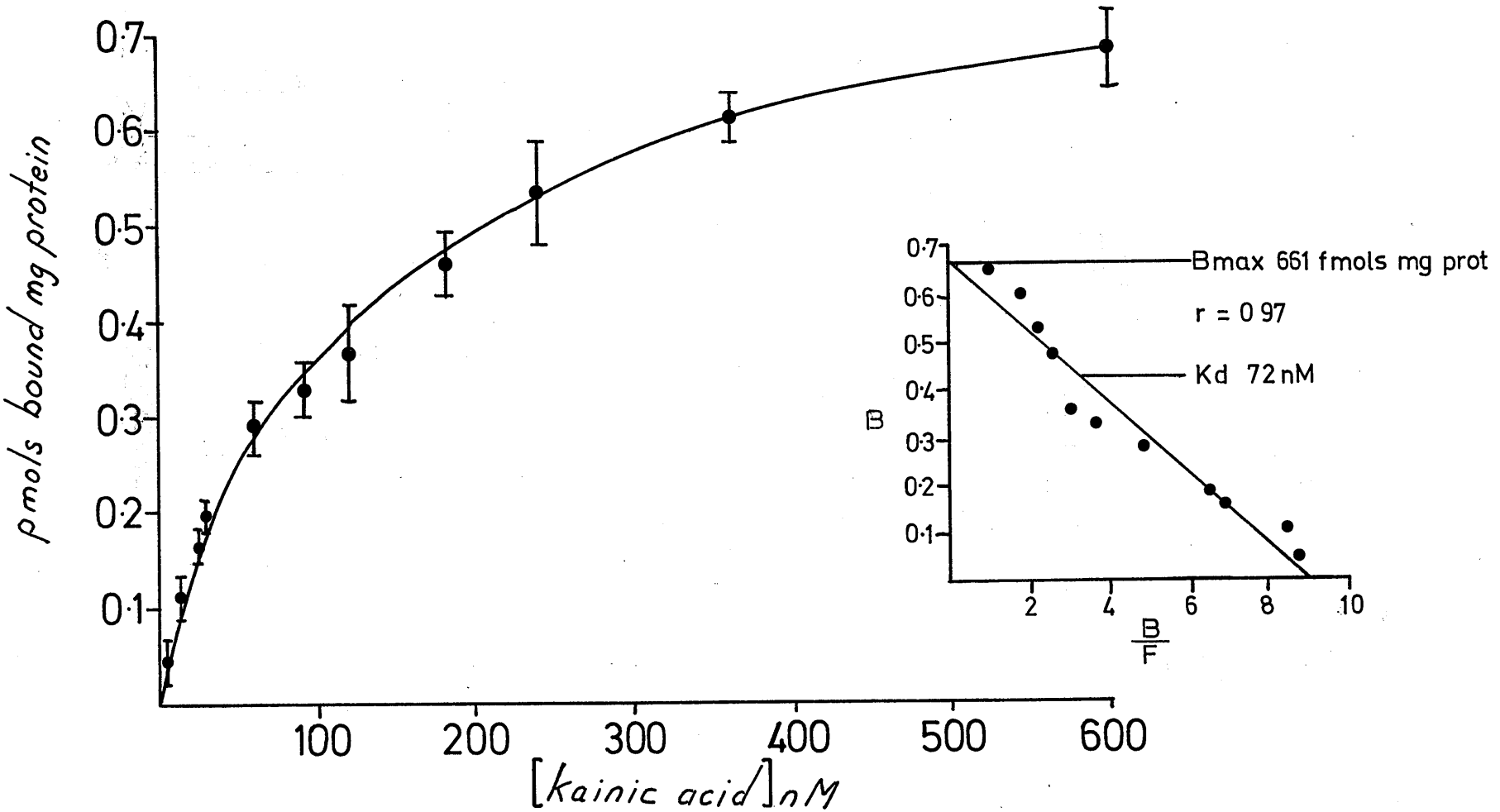
Results

A saturation curve of the binding is shown in Figure 5.10. Although non-specific binding was linear over the concentration range shown it represents a high proportion (approximately 70%) of the total binding. In addition there was considerable variation in the levels of binding to tissue prepared from different rats. The relatively small error bars are a reflection of the relatively high number (approximately N = 12) of separate determinations. The inset on Figure 5.10 shows a Scatchard plot of the same data and gives an estimate of the Kd = 72 nM and a βmax of 661.0 fmols/mg protein. Non-specific binding was estimated by the use of excess (0.1mM) cold Kainic acid, but curiously L-Glutamate at the same concentration of 0.1mM displaced only about 10% of the total binding. In addition it was not possible to demonstrate specific kainic acid binding to membranes prepared from rats less than 30 days old, despite the utilization of synaptic membranes of varying degrees of purity.

FIG 5.10

The specific binding of ^{3}H - Kainic Acid to cortical membranes prepared from adult (50 day old) rats. The assay was performed in KPO_4 buffer under conditions stated in the text. Kainic acid concentrations varying between 6-600nM were used to generate the saturation curve. The results are means $\pm$ SEM of 8 - 10 separate experiments. The kinetic parameters of the binding were estimated from a Scatchard plot of the saturation curve data which is shown as an inset on the Figure.

FIG 5₁₀


0·7
0·6
0·5
0·4
0·3
0·2
0·1
pmols bound/mg protein
100
200
300
400
500
600
[kainic acid]nM
B
0·7
0·6
0·5
0·4
0·3
0·2
0·1
2
4
6
8
10
B/F
Bmax 661 fmols mg prot
r = 0 97
Kd 72 nM

Discussion

Although Figure 5.10 shows that binding sites for kainic acid are present at very low concentrations and exhibit a high affinity for their ligand, in keeping with binding to a postsynaptic receptor, kainate itself is not a constituent of brain. Its relevance lies in the possibility that it could be binding to receptors for the natural endogenous ligand glutamic acid. If this is the case, then it would be expected that glutamate would be equally as effective as kainate in displacing bound radiolabelled KA; However, this was not found. This observation is in contrast to the report by Simon et al (1975), although these authors did report that binding sites for glutamate were 10 times in excess of those for kainate. This report is in broad agreement with my own studies which show a density of glutamate binding sites of 6.4pmols/mg protein compared with 0.661 pmols/mg protein for kainate.

If glutamate is acting as a major excitatory transmitter then it could be expected that neurons would possess receptors for the amino acid at a fairly early stage in development. The inability to demonstrate KA binding to membranes prepared from young rats is therefore curious. One possible explanation could be that KA may be binding to a small subpopulation of glutamate receptors that only become apparent later on in development. It has been reported that neurons in the striatum of very young rats are not susceptible to the neurotoxic effects of injected kainic acid (Campochiaro and Coyle, 1978).

Subsequent experiments reported in Section 5.4 demonstrated that KA was ineffective as a displacer of ^{3}H-L-Glu bound in the absence of Na^{+}, inhibiting only 5% of the glutamate binding at a KA concentration of

10^{-4}M. Thus, it seemed unlikely that KA receptors are synonymous with those binding glutamate.

There have been many other recent reports of KA binding to membranes prepared from various brain regions (Schwarcz and Fuxe, 1979; London and Coyle, 1979; Vincent and McGeer, 1979) all exhibiting similar kinetic constants. London and Coyle (1979) reported a duplicity of binding sites for KA in rat forebrain membranes, one with a very high affinity for KA with a Kd of 2.8 nM and a B_{max} of 0.4 pmols/mg protein. Because all the reported kinetic parameters of KA binding show a 20-30 fold difference to those characterizing glutamate binding, it is probable that two different populations of receptors are being measured. This view is strongly supported by a report that binding sites for kainate and glutamate can be separated following treatment of membranes with cholate (Michaelis et al, 1980). The same authors also report that a reduction of the isopropylene side chain of kainic acid strongly decreases its affinity for the binding sites. This would be unexpected if KA was binding to the same 3 point attachment site thought to bind the 2 charged carboxyl groups and the amino group of glutamate (and Kainate). A possible explanation for this observation however, could be that a distinct KA receptor possesses a lipophilic region which interacts with the hydrophobic unsaturated KA side chain and thus stabilizes the ligand-receptor complex. This strengthens the suggestion by Roberts (1981) that KA receptors may be binding sites for an, as yet, unidentified endogenous ligand.

Further insight into the possible site of action of KA comes from neuroanatomical studies of the toxic effects of KA. The neurotoxic potency of amino acids parallel their ability to depolarize neuronal cell membranes, and on this basis a excitotoxic hypothesis of neuronal death has bee

suggested (Olney et al.,1974). The postulate is that unphysiological concentrations of the amino acids cause a continuous depolarization and a sustained increase in membrane permeability - this in turn would lead to ionic changes of such magnitude that the normal homeostatic mechanisms designed to maintain the resting potential (i.e. the membrane bound Na^+/K^+ ATPase) could not restore the balance. Cell death would be the result of drastic shifts in the intracellular ionic balance.

If glutamate receptors are responsible for mediating the neurotoxic effects of KA then areas receiving glutamatergic projections should be particularly susceptible. Although this seems to be the case in the cerebellum, retina, pyriform cortex and hippocampus (McGeer & McGeer,1981), neuronal degeneration evoked by KA in the striatum is highly dependent on the presence of an intact glutamate projection pathway from the cortex (McGeer and McGeer, 1978). This is not consistent with a direct interaction of KA with postsynaptic glutamate receptors and has led to the suggestion that KA may be acting presynaptically to cause glutamate release. Such an effect has indeed been reported by Cox and Bradford (1978). Alternatively, KA could be responsible for maintaining elevated levels of glutamate at the synapse by blocking its re-uptake into glia or nerve endings. In this case KA would be expected to inhibit the Na^+ dependent high affinity binding of glutamate to membrane preparations. Reports here are conflicting. Lakshmanan and Padmanaban (1974) and McGeer et al (1981) both report such an inhibition whilst Roberts and Watkins (1975) and Balcar and Johnston (1972) find no such effect. My own studies do not show any inhibition by KA of Na^+ dependent glutamate binding. In addition, Na^+ independent binding of glutamate is not affected by KA at least in the cortex (Sanderson and Murphy, 1981).

A consideration of all these data, together with evidence accumulated in recent years by Watkins and his colleagues that shows that pharmacological agents have different inhibitory potencies towards glutamate and KA induced excitation, is suggestive of a presynaptic or extra junctional site of action for KA. So KA could be activating receptors for a glutamate-like endogenous ligand, which normally modulates the release of glutamate. Because of the conflicting evidence in the literature though, some effect on uptake cannot be ruled out. It is also possible to hypothesise alternative mechanisms for the action of KA consistent with the experimental findings. For example, KA may be binding to ion channels in the presynaptic membranes that mediate the flow of Ca^{++} into the nerve endings. Or it may be affecting the activity of the low affinity glutamate transport site which may also play a crucial role in regulating synaptic levels of glutamate (and aspartate).

Discrimination between these modes of action is very difficult to assess at the present time, although very recent studies have been directed towards identification of a natural ligand for the KA binding site. A strong candidate for such a ligand emerged from a report that the naturally occurring folic acid derivative methyltetrahydrofolate (MTHF) is a potent and specific competitor for KA binding sites in rat cerebellar membranes (Ruck et al, 1980). These binding study results have not, however, been confirmed by recent studies of the neurotoxic and neurophysiological effects of MTHF. Although injection of folates into various brain regions produced brain damage, this damage was at sites somewhat distant from the site of injection and the folates did not mimic the specific local neurotoxic effects of KA (Olney et al, 1981). Similarly neurotoxic effects of MTHF in the cerebellum are much less pronounced than those evoked by KA (Roberts et al, 1981). Biochemical studies by the same

authors have demonstrated, in addition, that MTHF, unlike KA, did not stimulate an increase in cerebellar cyclic GMP levels. Very recent neurophysiological studies have also shown that MTHF does not evoke excitatory PSP's in the olfactory cortex, although KA is a potent agonist at these sites. The search continues!

5.5 Aspartate Binding

Much of the work directed towards elucidating a role in synaptic transmission for the dicarboxylic amino acids has concentrated on defining the neurochemical effects and properties of glutamate. The neurophysiological evidence, however, favours the candidature of aspartate no less than glutamate. Further support comes from studies of the specific release of aspartate from many brain areas, of the high affinity uptake of aspartate into CNS tissue, and more recently from neuropharmacological studies of the effects of amino acid analogues on the excitatory properties of aspartate (see Johnson, 1978; and DeFeudis, 1979, for reviews).

Simply from theoretical considerations it seemed possible that if both amino acids had a role in synaptic transmission in the CNS, and particularly in the cortex, much of the effect might be mediated through shared or at least synergistic sites of action on the post synaptic cell.

Because of the lack of effect of glutamate (as well as aspartate) analogues on the binding of L-Glu to cortical membranes, some level of interaction of glutamate with aspartate binding sites could not be conclusively ruled out. Indeed a lack of some cross - reactivity would be surprising in view of the probable ability of glutamate to alter its shape to fit different 3 point attachment sites. Equally interesting is the reported finding (section 5.4) that although aspartate did not affect ^{3}H-L-Glu binding (Na^{+} independent) to cortical membranes prepared from 50 day old rats, its affinity for these Na^{+} independent sites at 20 days was 2 times higher than that of glutamate.

It therefore seemed that an investigation of the interaction of L.Asp to cortical membranes using the binding assay may provide answers to these questions:-

1. Does ^{3}H-L-Asp bind to cortical membranes with kinetic parameters in keeping with a proposed neurotransmitter function for this amino acid?

2. Do any such binding sites represent a unique population of sites specific for aspartate or is there a degree of overlap with sites that bind glutamate?

3. Are there any changes in the structural requirements of these sites throughout development?

The assay conditions used to estimate aspartate binding were the same as those used for glutamate binding. Thus an assumption was made that due to the fact that glutamate and aspartate are very similar in terms of shape, structure and chemical reactivity the conditions necessary to demonstrate glutamate binding to cortical membranes would also hold for aspartate.

Results

Figure 5.11 shows the increase in specific binding of aspartate/mg protein under Na^{+} free conditions to membranes prepared from 50 day old rats. The concentration range used varied between 0.1 - 2.0μM ^{3}H-L-Asp. This binding saturates at a concentration of about 1.2μM Asp. As can be seen from the graph, non-specific binding does not saturate over the concentration range used, but increases linearly with a correlation coefficient of 0.99. This non-specific binding represented about 30% of the total binding - a smaller proportion than in the glutamate binding assay. Replotting these data from the saturation curve as a Scatchard plot (Figure 5.12) provides an estimate

FIG. 5II

The specific Na^+ independent of ^3H-L-Aspartate to cortical membranes prepared from adult (50 day old) rats. Non-specific binding is also shown on the graph. Binding was estimated at L-Asp concentrations varying between 0.1 - 2.0μM. The results for specific binding are mean $\pm$ SEM of 5-6 separate experiments.

FIG 5.11

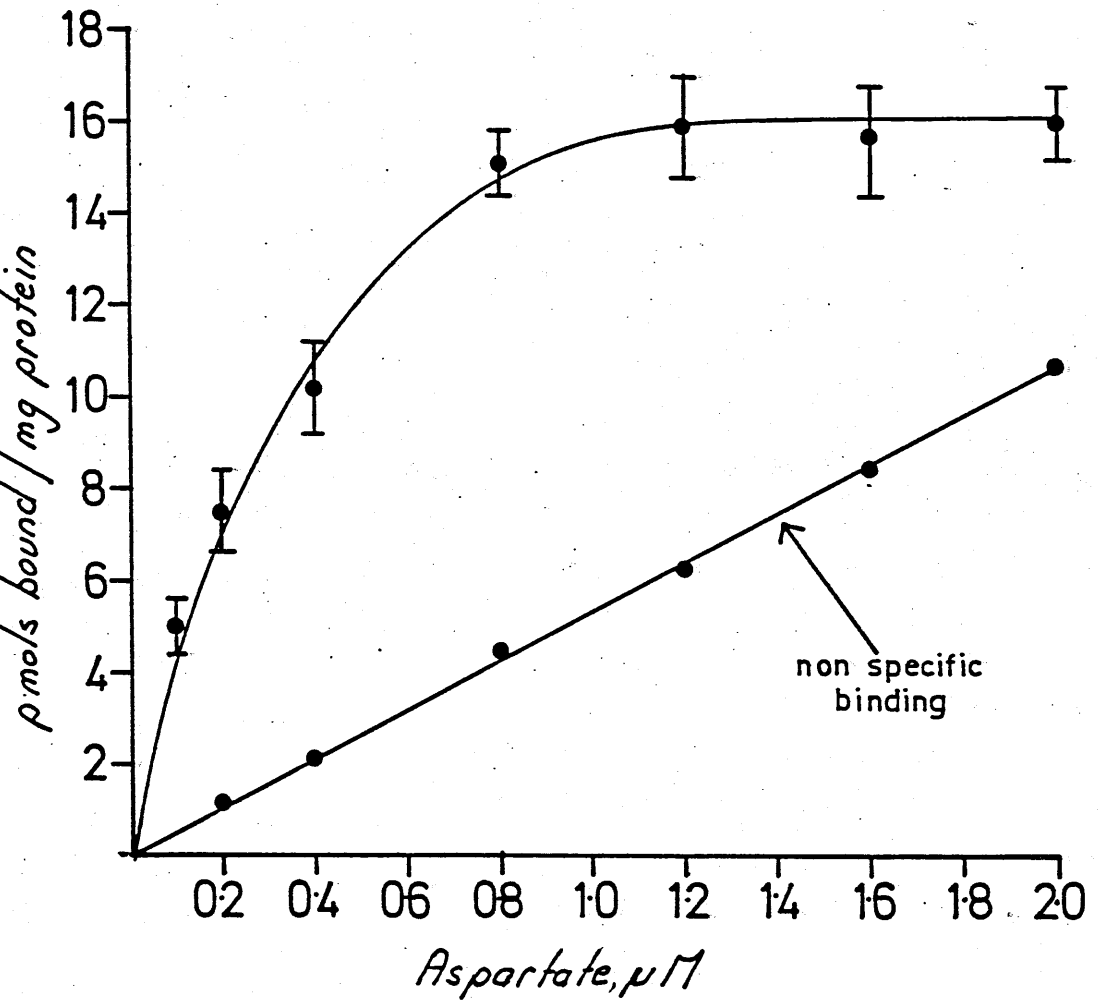
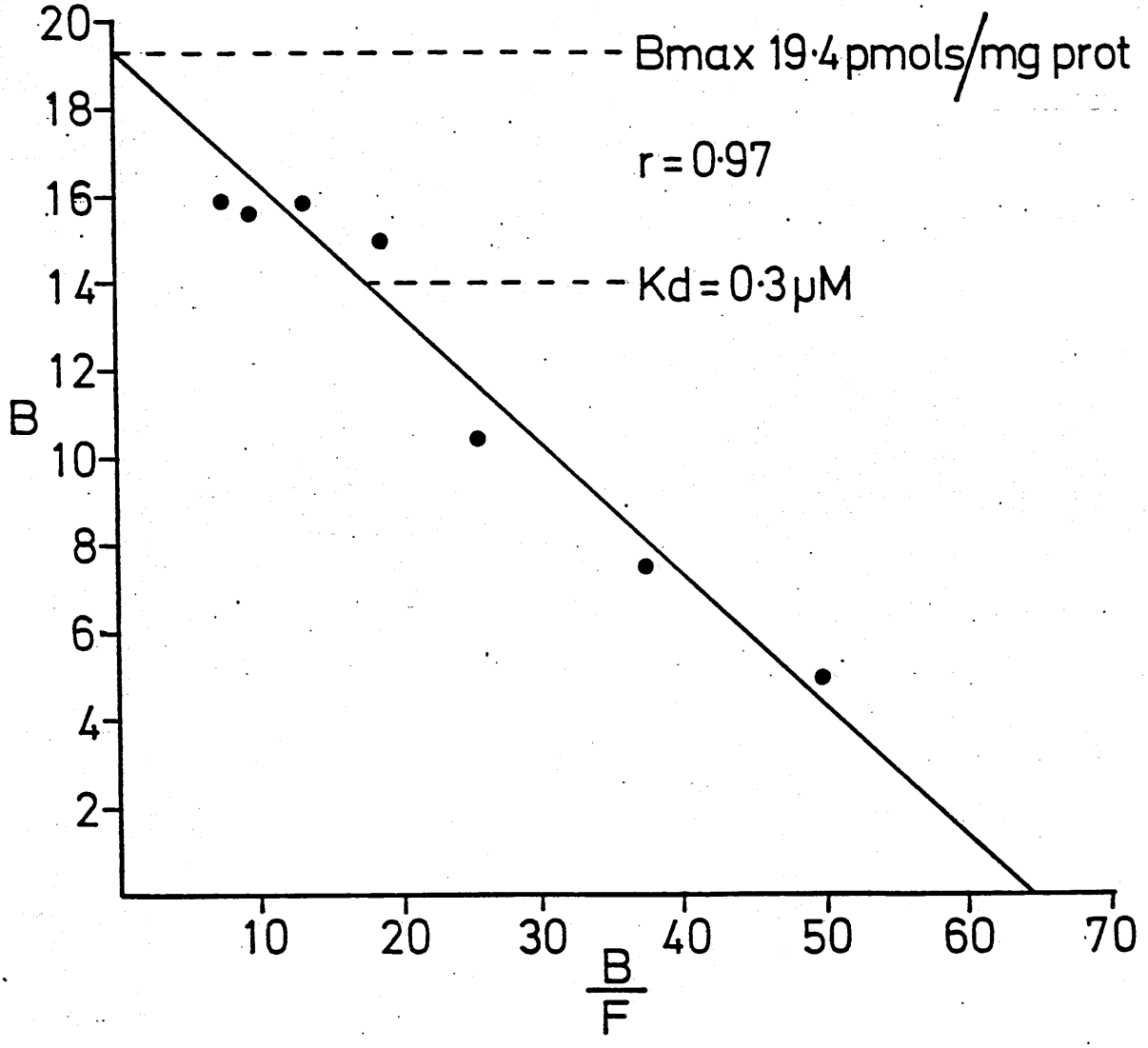

18
16
14
12
10
8
6
4
2
p mols bound / mg protein
non specific binding
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
1·8
2·0
Aspartate, μM

FIG. 5.12

Scatchard plot of the saturation isotherm shown in Fig. 5.9 describing the Na^+ independent binding of ^{3}H-L-Asp to adult rat cortical membranes.

FIG 5₁₂


Bmax 19·4 pmols/mg prot
r = 0·97
Kd = 0·3 μM
B
20
18
16
14
12
10
8
6
4
2
10
20
30
40
50
60
70
B/F

of the Kd as 0.3μM ± .026 and a Bmax of 19.4 ± 1.6 p mols/mg protein.

The specific binding in the presence of 50 mM Na^+ is shown in Figure 5.13, and again is saturable over the concentration range used, although at much higher levels of binding. Non-specific binding is again linear. A Scatchard plot (Figure 5.14) reveals a Kd of 1.5 ± .09μM and a Bmax of 361 ± 18.4 pmols/mg protein.

Similar studies of the binding of ^{3}H-L-Asp to membranes prepared from 20 days old rats gave very different results, however. Figure 5.15 shows the binding isotherm in the absence of Na^+. Specific binding was linear over the same concentration range used to generate the saturation curve in adult animals (r^2 = 0.95) and only saturated at concentration of L-Asp up to 8mM. Not surprisingly, a Scatchard analysis of this data (shown as Fig. 5.16) revealed very different kinetic parameters than shown for the same site in adult animals. The site density increased to a value of 54.5 ± 2.8 p mols/mg protein and the affinity of L-Asp for this site was much lower with a Kd of 6.9 ± 0.42μM.

Na^+ dependent binding also exhibited different characteristics in 20 day old animals than in 50 day old animals. Figure 5.17 shows the binding curve over the concentration range 0.2 - 2.0mM. As in the adult, binding does begin to saturate over this range although non-specific binding rises linearly. A Scatchard plot (Fig. 5.18) however gives an estimate of the Kd as 3.26 ± .08μM (compared to a Kd of 1.5μM in the adult) and a site density of 1157 ± 37 pmols/mg protein i.e. approximately 3 times higher than in adults.

To see at which stage this transition to adult kinetics might occur, a further study of ^{3}H-L-Asp binding to cortical membranes prepared from 30 day old rats was carried out, again in the presence and absence of Na^+.

FIG. 5-13

The specific Na^+ dependent binding of ^{3}H-L-Asp to cortical membranes prepared from adult (50 day old) rats. The binding assay was carried out under the conditions described in the text. Binding was estimated at L-Asp concentrations varying between 0.2 - 2.0μM. Non-specific binding is also shown on the graph. The results for specific binding are mean $\pm$ SEM of 6-8 separate experiments.

FIG 5₁₃

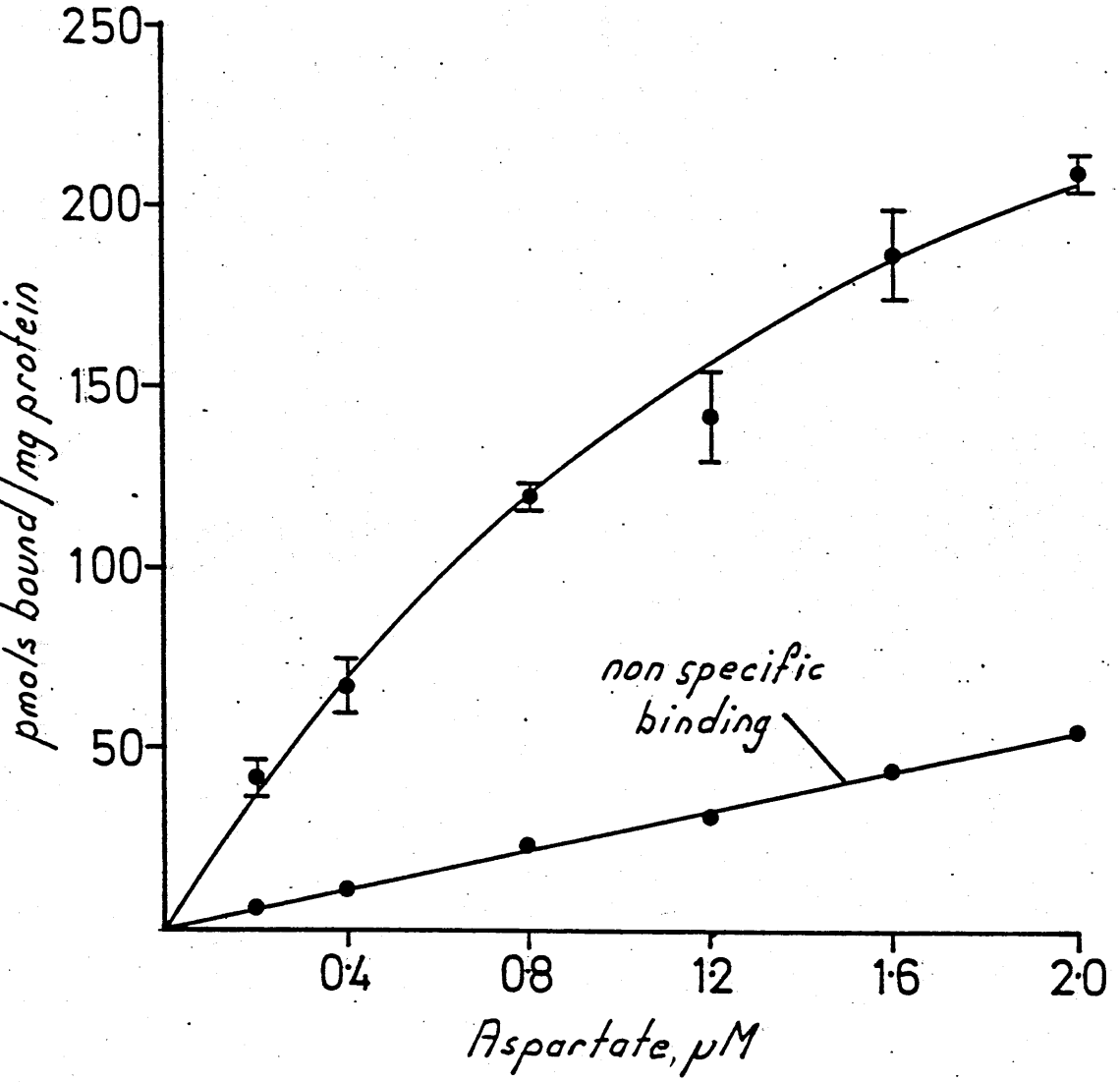

250
200
150
100
50
pmols bound/mg protein
non specific binding
0·4
0·8
1·2
1·6
2·0
Aspartate, μM

FIG. 514

Scatchard plot of the saturation curve shown in Fig. 513 describing the Na^+ dependent binding of ^3H-L-Asp to adult rat cortical membranes.

FIG 5₁₄

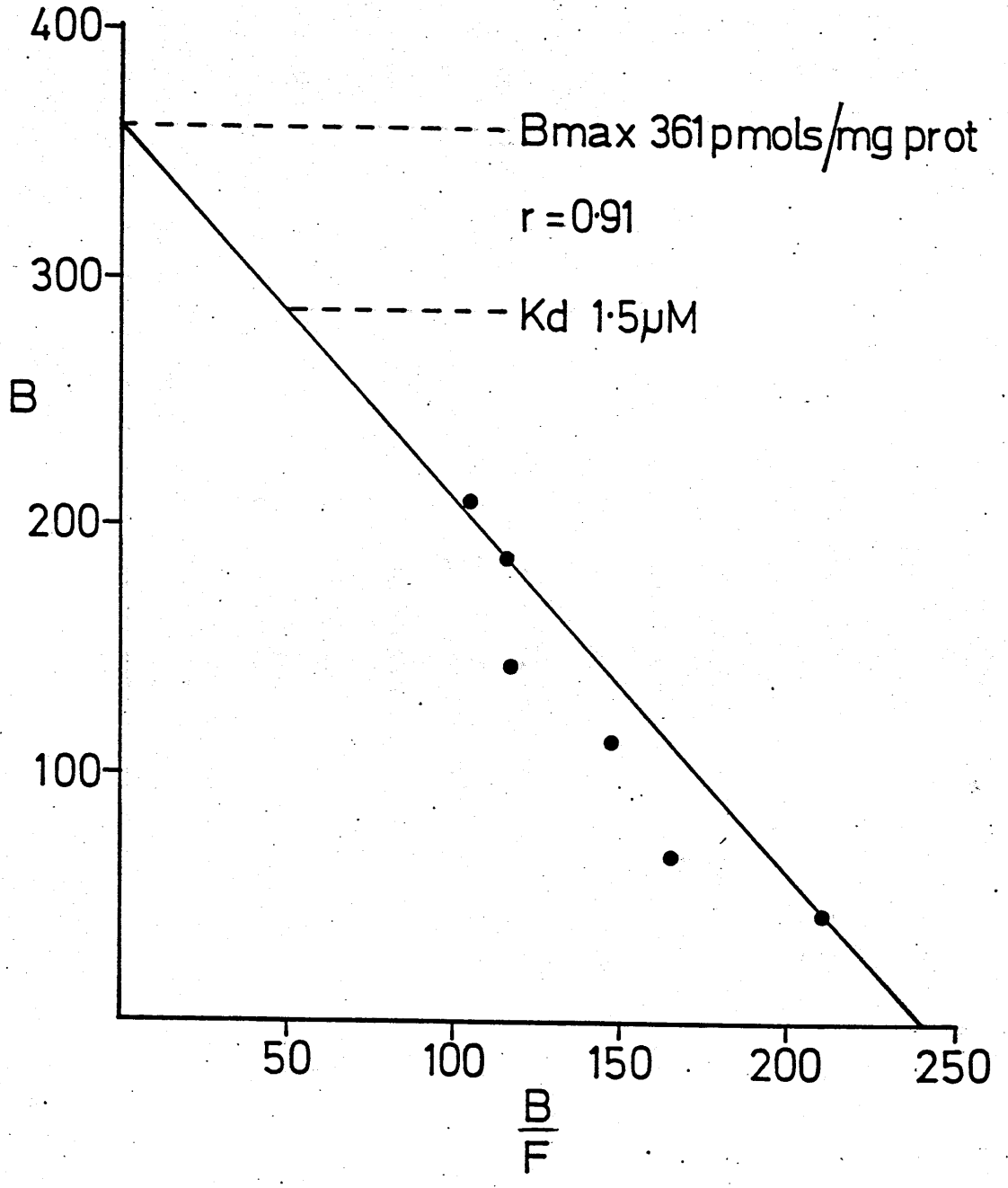

400
300
200
100
B
Bmax 361 pmols/mg prot
r = 0·91
Kd 1·5μM
50
100
150
200
250
B/F

FIG. 515

Saturation curve showing the specific Na^+ independent binding of ^{3}H-L-Asp to cortical membranes prepared from 20 day old rats. Concentrations of L-Asp varying between 0.1 - 8.0 μM were used to generate the binding isotherm. The results are means $\pm$ S.E.M. of 4 (estimates at 4.0 μM and 8.0 μM) and 8 (all other concentrations) separate experiments.

FIG. 516

Scatchard plot of the saturation curve shown above.

FIG 5.15

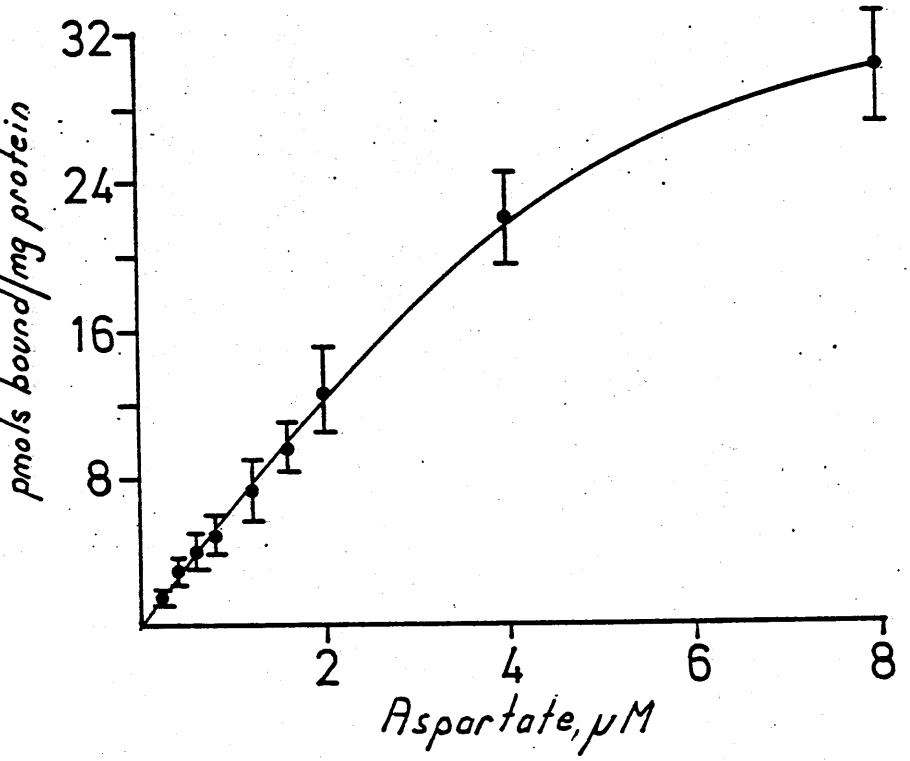


FIG 5.16

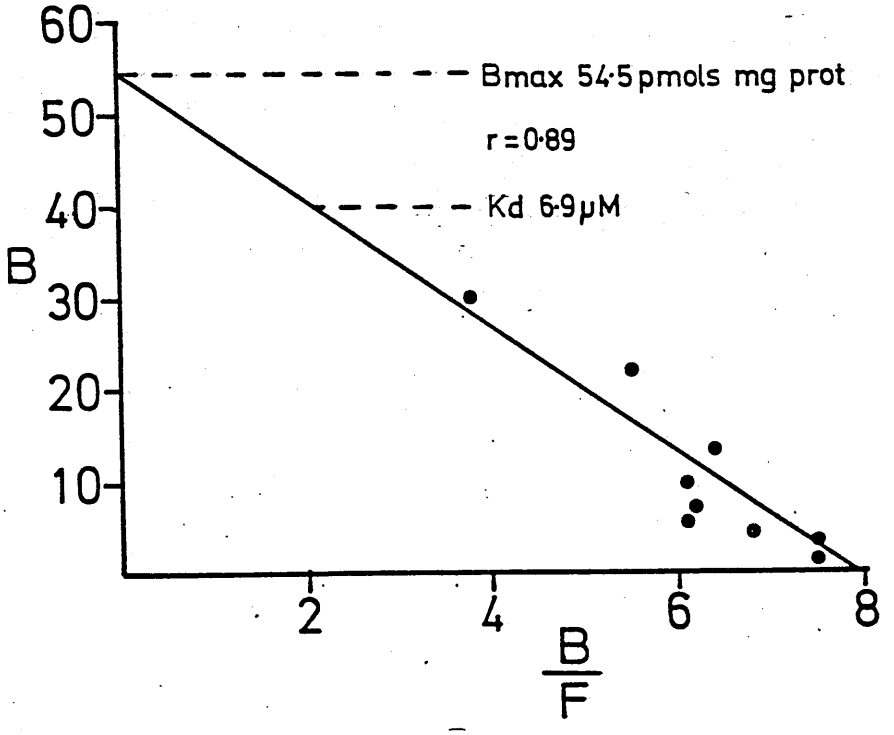


FIG. 517

Saturation curve showing the specific Na^+ dependent binding of ^{3}H-L-Asp to cortical membranes prepared from 20 day old rats. Non-specific binding is also marked on the graph. The results for specific binding are mean ± SEM of 6-8 separate experiments.

FIG. 518

Scatchard plot of the saturation curve shown above.

FIG 5.17

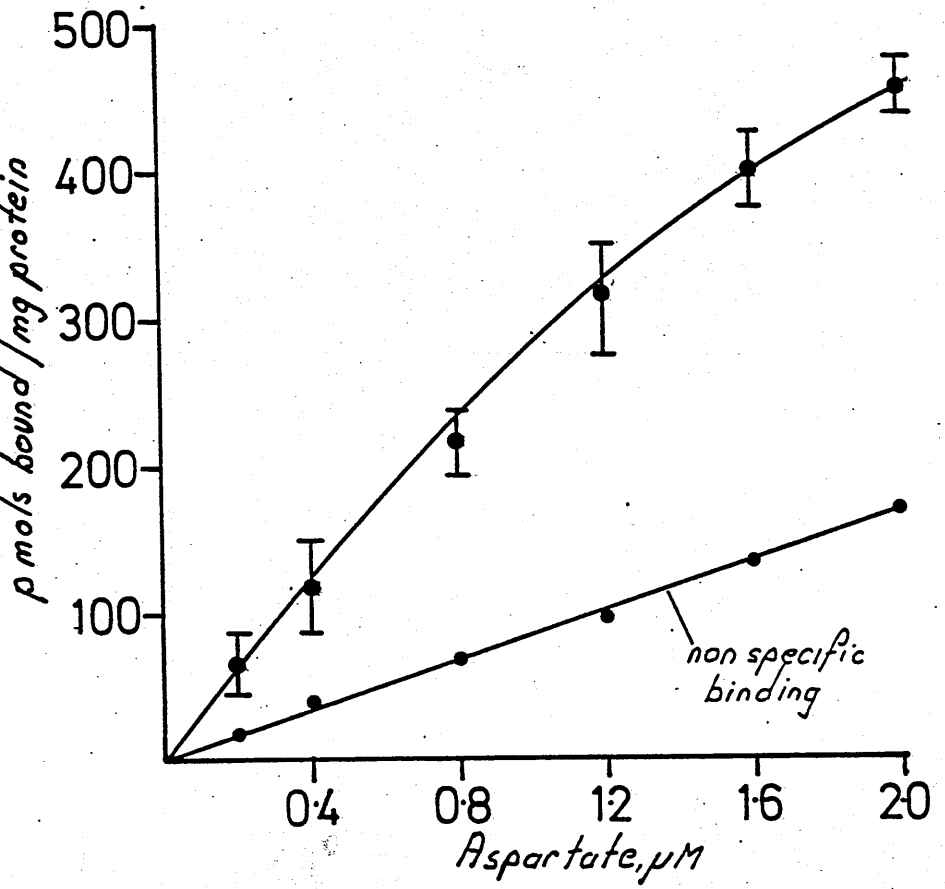


FIG 5.18

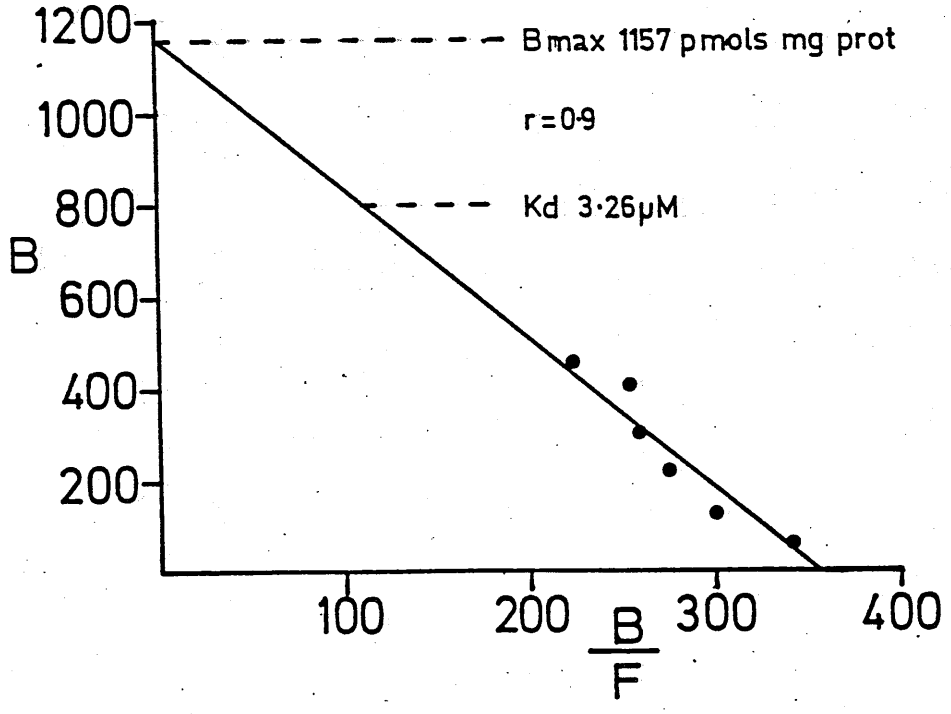


Figure 5.19 & 20 shows that both the saturation isotherm and the Scatchard plot for Na^+ dependent binding are practically indistinguishable from those found in the adult, generating an estimate of kd = 1.55 μM (1.5 μM in the adult) and a B max of 378 pmols/mg protein (361 pmols/mg protein in the adult.)

It was much more difficult to get any estimate of the Na^+ independent binding of ^{3}H-L-Asp to cortical membranes prepared from 30 day old animals. Results from many experiments gave widely different results. These variations were not due to different laboratory procedures as considerable efforts were made to standardize the preparation of the tissue and the binding assay protocol. It is possible that they represent real differences between individuals, if this stage coincides with a time when biochemical mechanisms subserving neurotransmission are being activated or modified in response to environmental stimulation. A large sample size, coupled with a meticulous assay technique, allowed for the estimation of the kinetic parameters of Na^+ independent L-Asp binding at 30 days of age, as well as at the 35 and 45 day age points. The latter two ages proved easier to investigate! The results of these determinations are shown on Table 5.4. They show that there is no significant change in the kd value of the binding site for L-Asp from 30-50 days, but that the Bmax increases 3-fold during this time.

Because of a growing interest in the structural requirements of amino acid binding sites, particularly throughout ontogeny, the ability of aspartate and glutamate analogues (with the addition of D-Asp) to displace ^{3}H-L-Asp bound to both the Na^+ independent and Na^+

FIG. 5·19

Saturation curve showing the specific Na^+ dependent binding of ^3H-L-Asp to cortical membranes prepared from 30 day old rats. Non-specific binding is also shown on the graph. The results are means ± SEM of 6-8 sep rate experiments.

FIG. 5·20

Scatchard plot of the saturation curve shown above.

FIG 5₁₉

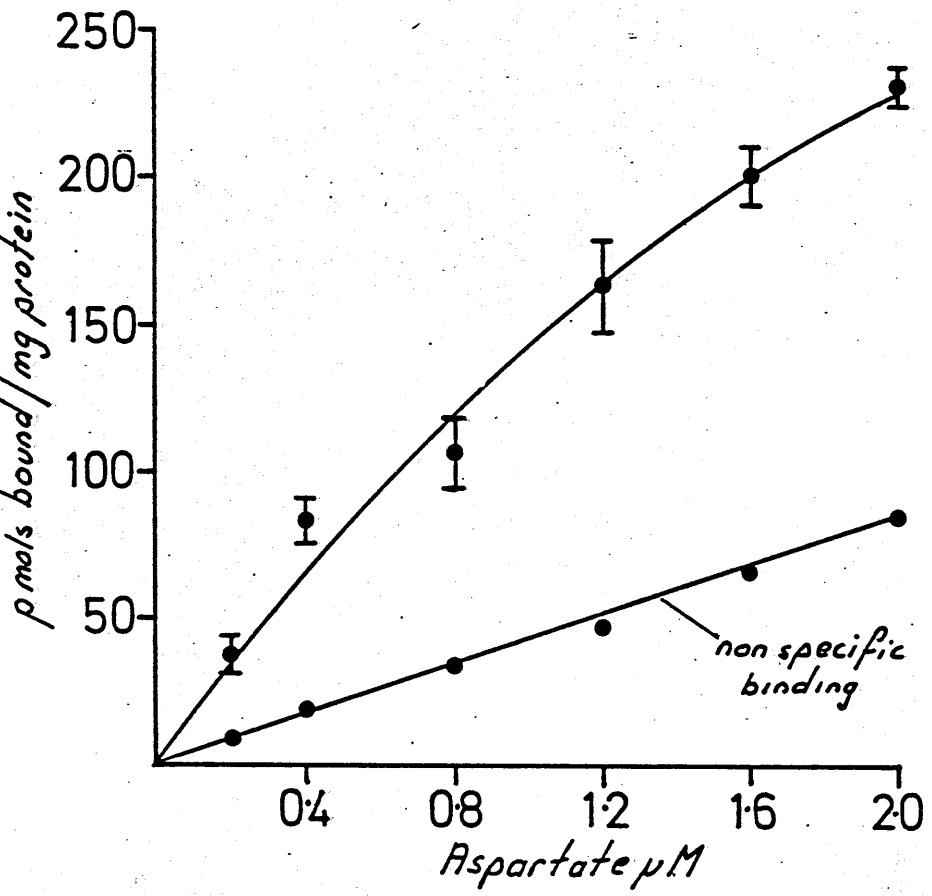
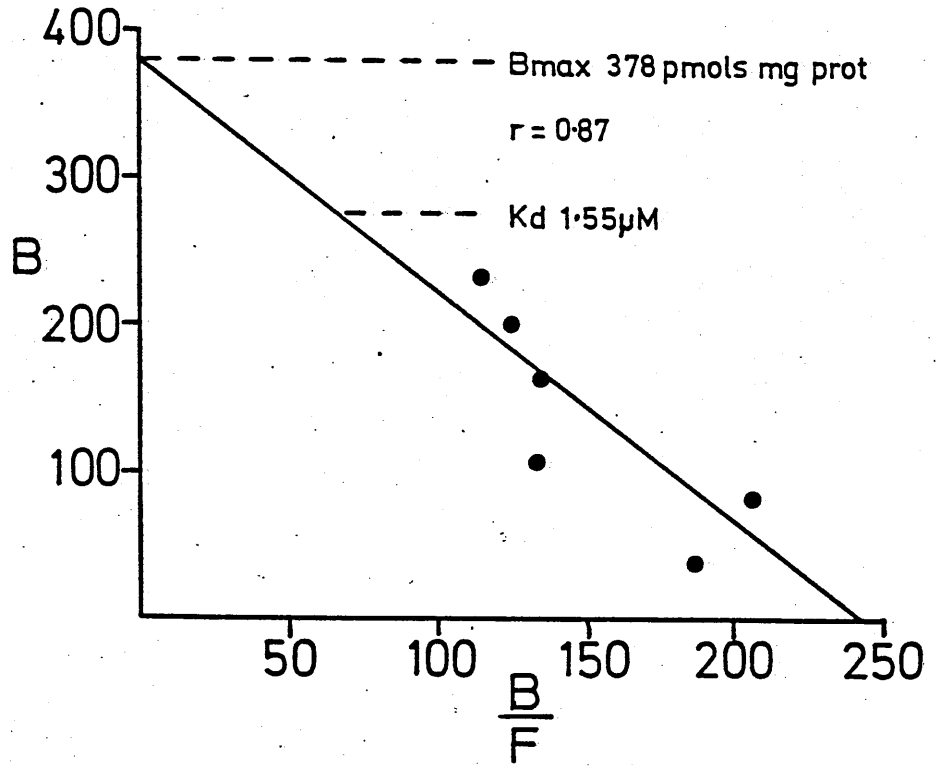


FIG 5₂₀



dependent sites was investigated at the 50 day and 20 day age points.

The results of these displacement studies are shown in Table 5.5. IC50 values were again measured from log dose/percent inhibition plots. Shown in Figs. 5_{21} and 5_{22} for 50 day and 20 day respectively.

Considering first the displacement of L-Asp bound to the Na^+ independent site in adult rats, it can be seen that this site is strictly stereospecific for the L-isomer of aspartate. D-Asp did not displace any of the binding at all at a concentration of 10^{-4}M. Inhibition of the specific binding of L-Asp by L-Glu was approximately 30% at a glutamate concentration of 10^{-4}M. D-Glu was however quite effective as a displacer of bound ^{3}H-L-Asp, although with a potency about 14 times weaker than that of L-Asp itself. The potency of D-Glu was matched by the aspartate agonist NMDA and the aspartate antagonist DαAA. In contrast, the proposed glutamate agonist kainic acid was totally ineffective at displacing this binding, as was the glutamate antagonist GDEE.

The pattern of the effectiveness of these compounds in displacing ^{3}H-L-Asp bound to the Na^+ independent site at 20 days was very different however. L-Glu was equally as effective as L-Asp in displacing the L-Asp binding at this age point with respective IC50's of 2.0μM and 5.0μM. In addition the conformationally restricted glutamate analogue kainic acid showed some affinity for the Na^+ independent site in these younger animals with an IC50 of approximately 10μM; very similar to that of the specific aspartate analogues NMDA and DαAA.

The Na^+ dependent site in contrast showed very little change in the comparative affinity for the different analogues between 20-50 days.

TABLE 54

The kinetic parameters describing the specific Na^+ independent binding of ^{3}H-L-Asp to cortical membranes prepared from rats between 30 - 50 days of age. The results show the mean ± S.E.M. of the number of experiments shown in brackets after each value.

Age (days)	Kd (μM)	B Max (pmols/mg protein)
30	0.29 ± 0.018 (12)	6.6 ± 0.8
35	0.31 ± 0.026 (11)	14.8 ± 2.1
45	0.27 ± 0.02 (8)	18.6 ± 1.14
50	0.30 ± 0.026 (6)	19.4 ± 1.6

FIG. 521

Inhibition of the binding of L-^{3}H-Aspartate by amino acid analogues on membranes prepared from the cerebral cortex of 50 day old rats:

A - Inhibition of Na^{+} independent binding.

B - Inhibition of Na^{+} dependent binding.

■ L - Aspartate

□ L - Glutamate

○ D - Aspartate

△ N - Methyl-D-Aspartate

● DL and Amino-adipate

Kainic Acid and Glutamate-diethyl-ester were ineffective at displacing the binding at the highest concentration tested (10^{-4}M).

Each point is the mean of 4-6 separate experiments which did not differ by more than 10%. The results of these displacement studies with ^{3}H-L-Asp are summarized in Table 55.

FIG 5$_{21}$

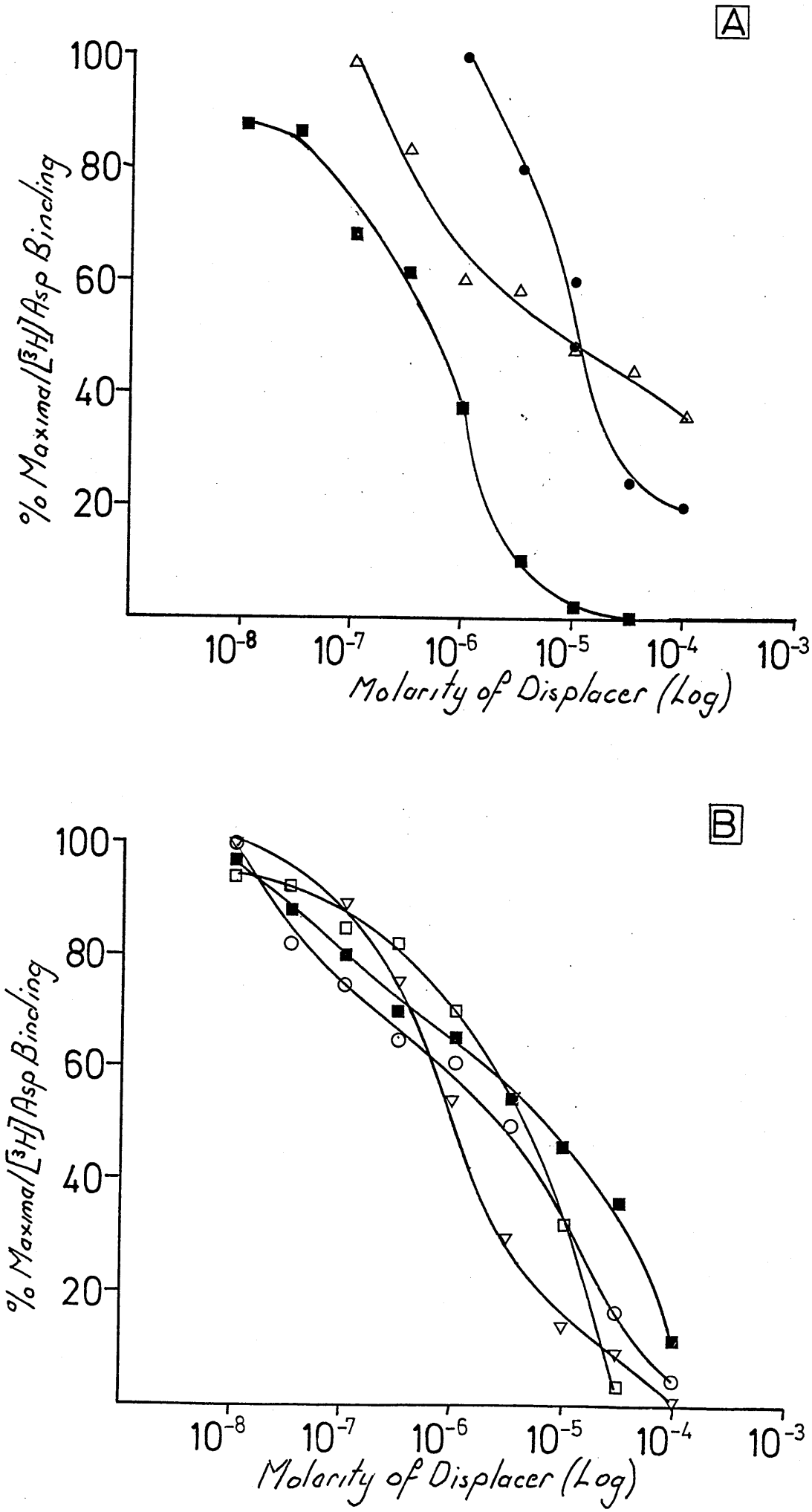

A
100
80
60
40
20
% Maximal [3H] Asp Binding
10-8
10-7
10-6
10-5
10-4
10-3
Molarity of Displacer (Log)
B
100
80
60
40
20
% Maximal [3H] Asp Binding
10-8
10-7
10-6
10-5
10-4
10-3
Molarity of Displacer (Log)

FIG. 5_{22}

Inhibition of the binding of ^{3}H-L-Aspartate by amino acids to membranes prepared from the cortices of 20 day old rats.

A - Inhibition of Na^{+} independent binding.

B - Inhibition of Na^{+} dependent binding.

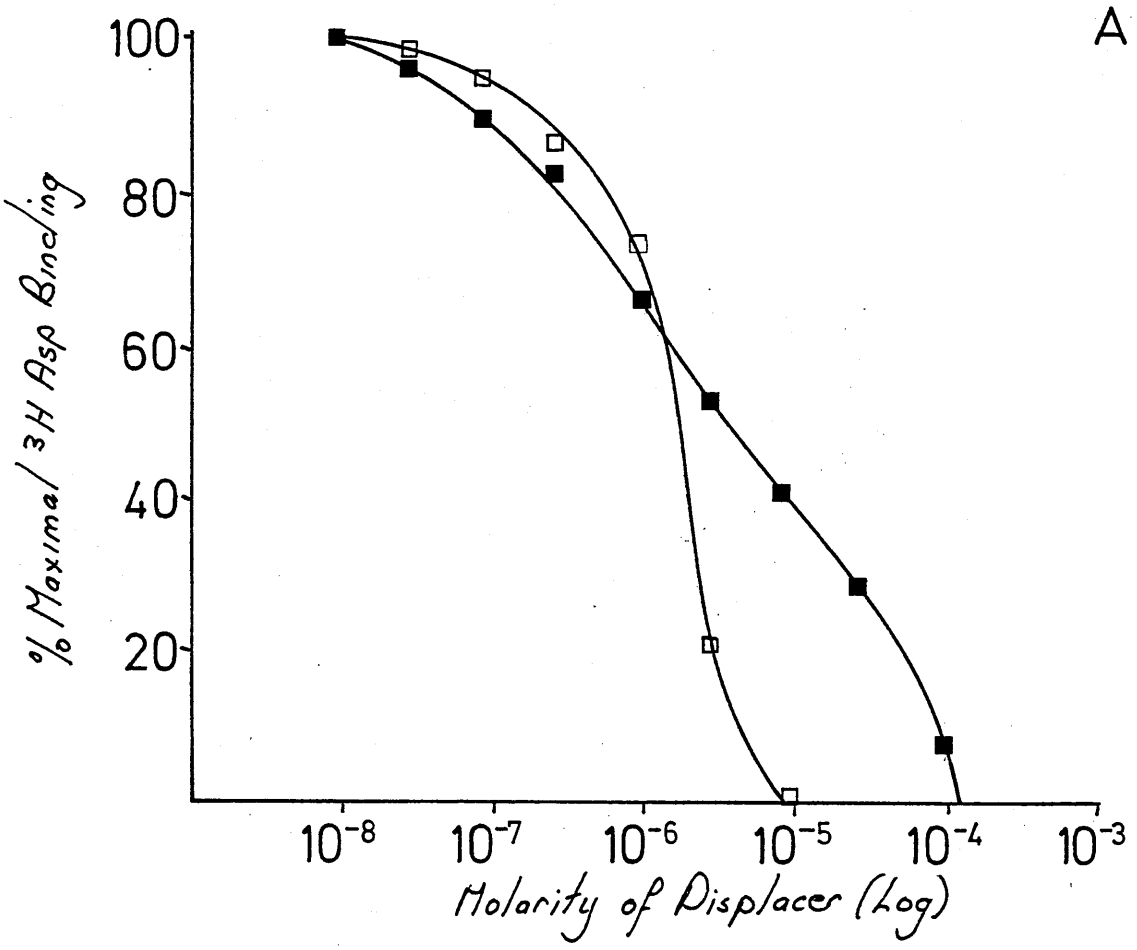
- L - Asp.
- L - Glu.
- D - Asp.

All other amino acid analogues tested were ineffective as displacers of ^{3}H-L-Asp binding at the lowest concentration tested (10 - 4 M).

Although the IC_{50} for the inhibition of the Na^{+} independent binding are very similar for L-Glu and L - Asp, L-Glu is effective over a much narrower concentration range than L-Asp.

Their ability to displace Na^{+} dependent ^{3}H-L-Asp binding is very similar in the 20 day old animal.

FIG 522


A
100
80
60
40
20
% Maximal 3H Asp Binding
10-8
10-7
10-6
10-5
10-4
10-3
Molarity of Displacer (Log)

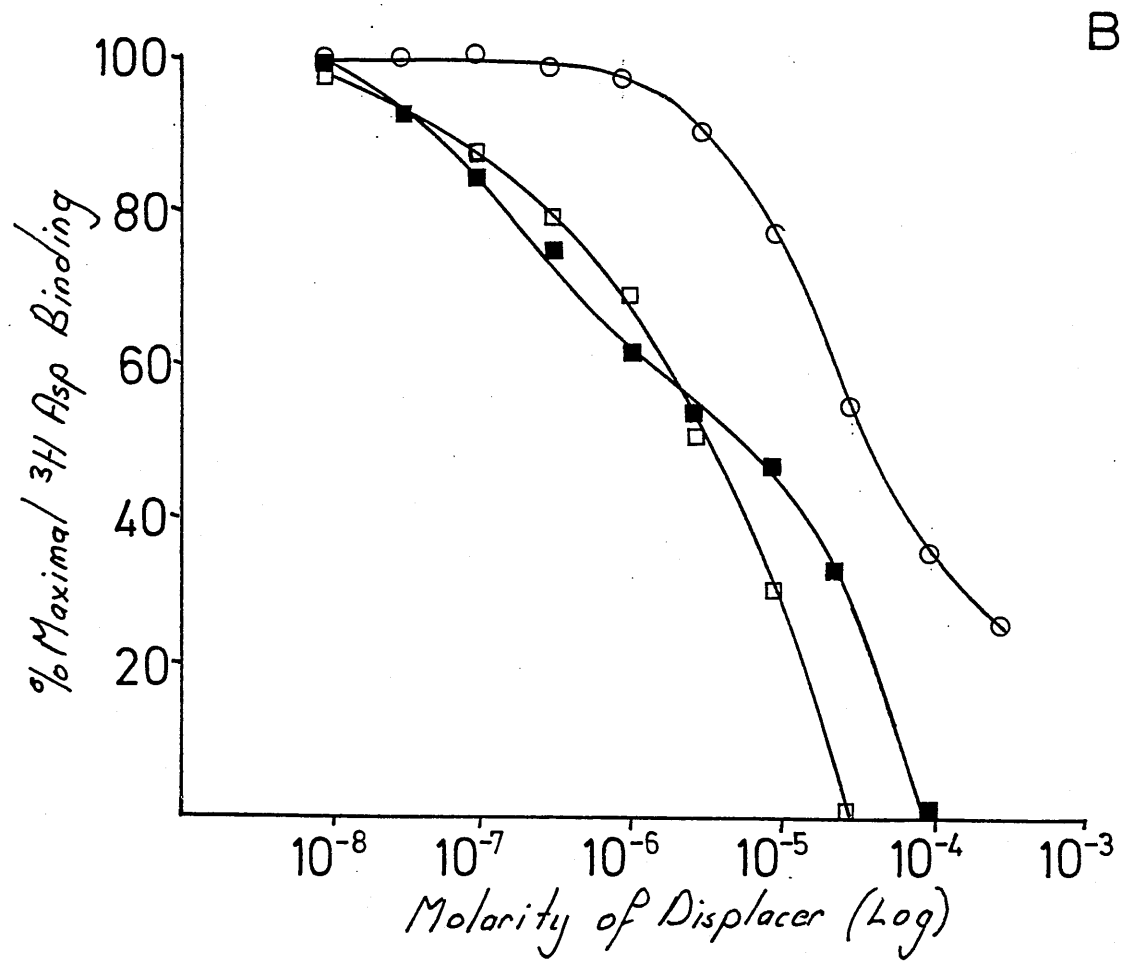

B
100
80
60
40
20
% Maximal 3H Asp Binding
10-8
10-7
10-6
10-5
10-4
10-3
Molarity of Displacer (Log)

TABLE 5.5 IC50 values of amino acid analogues (concentration range 10^{-9} - 10^{-4} M) acting as displacers of ^{3}H-L-Asp (0.6μM) specifically bound to membranes prepared from the rat cerebral cortex at 20 and 50 days.

	50 DAY		20 DAY	
Analogue	Na^+ Dep.	Na^+ Ind.	Na^+ Dep.	Na^+ Ind.
LAsp	8.4×10^{-6}	7.2×10^{-7}	8.0×10^{-6}	5.0×10^{-6}
LGlu	6.0×10^{-6}	$> 10^{-4}$	6.0×10^{-6}	2.0×10^{-6}
DAsp	5.0×10^{-5}	$> 10^{-4}$	5×10^{-5}	$> 10^{-4}$
DGlu	1.0×10^{-6}	$> 10^{-5}$	$> 10^{-4}$	$> 10^{-4}$
KA	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-5}$
GDEE	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$
NMDA	$> 10^{-4}$	$> 10^{-5}$	$> 10^{-4}$	$> 10^{-5}$
DLαAA	$> 10^{-4}$	$> 10^{-5}$	$> 10^{-4}$	$> 10^{-5}$

D-Glu was the exception, as this was a weak displacer of ^{3}H-L-Asp bound to the site at 20 days but was more effective than either L-Glu or L-Asp at 50 days. This Na^+ dependent site also showed some affinity for the D isomer of aspartate at both ages, but the potency of displacement was considerably less than for D or L-Glu or L-Asp. L-Asp and L-Glu were equally effective at displacing L-Asp specifically bound to the Na^+ dependent site at both age points.

Discussion

The presence of two high affinity binding sites for L-Asp on cortical membranes differing in their requirement for Na^+, is again consistent with a transmitter role for aspartate in the rat cortex. At the time of writing there has been only one report of the binding of L-Asp to tissue prepared from the rat cerebral cortex, although the binding assay was carried out using an isolated lipoprotein component extracted from synaptic membranes and not to whole membrane tissue. In that study, 3 binding sites for aspartate were reported (DePlazas and DeRobertis, 1976). The first, of very high affinity, has a Kd of 0.2 μM - very similar to the estimated affinity of the Na^+ independent site for aspartate described here. The Bmax was considerably higher however, at 280 pmols/mg protein (19.4 pmols, this study). This higher site density is undoubtedly due to the considerable increase in purity of the binding component in the tissue sample. This isolated lipoprotein also exhibited a medium affinity site(Kd 10 μM; βmax, 132 nmols) and a low affinity site(Kd 59 μm; β max 612 nmols). It is curious that the binding to the medium and low affinity sites was also in the absence of Na^+. It seems unlikely that these two sites could

be physiologically important in neurotransmission as a higher affinity and lower site density would be necessary for a postsynaotic receptor site, and a high affinity re-uptake site would be expected to show a strict requirement for Na^+. Alternatively, the independence from Na^+ could be due to a dissociation of the amino acid binding component of the recognition/transport molecule from the Na^+ binding site during the extraction and isolation procedure, although this would imply a fairly loose association within the membrane.

Sharif and Roberts (1981) have recently demonstrated ^{3}H-L-Asp binding to cerebellar synaptic membranes and found a single site that was Na^+ independent and gave a Kd of 0.874μM and a Bmax of 44 pmols/mg protein. Foster et al (1981) have also reported ^{3}H-L-Asp binding to membranes prepared from whole forebrain. This binding was also Na^+ independent with a Kd 1.26μM and a Bmax of 29 pmols, although they also report that this binding was increased 4 fold in the presence of 100mM Na^+ which would suggest the presence of a Na^+ dependent site as demonstrated in this report.

The structure of this Na^+ independent L-Asp binding site would seem to favour binding of substances that are aspartate-like in structure rather than glutamate-like. This is likely in view of the fact that the Aspartate analogues NMDA and DαAA can displace this binding with IC50 values of around 10^{-5}M. The IC50 value for L-Asp itself is 7.2×10^{-7}M and the binding is not displaceable by the glutamate analogues KA and GDEE nor by glutamate itself. Interestingly the results also show that D-Glu has some affinity for this Na^+ independent site. This is consistent with theoretical arguments reported earlier, that D-Glu might be a preferential agonist for amino acid receptors that are aspartate-preferring or that accept glutamate in a partially folded form. It is surprising however,

that L-Glu did not exhibit any affinity for this site in adults, as glutamate is a flexible molecule and it would be expected to be able to interact with aspartate binding sites in a partially folded form. These inhibition studies are very similar to those of DePlazas and DeRobertis (1976), who also showed a lack of effectiveness of L-glu in displacing binding from their high affinity site, whilst NMDA inhibited the binding by 45% at a concentration of 40μM. Their high affinity site was also stereospecific for the L isomer of aspartate.

These results are contradicted by Sharif and Roberts (1981) who reported that L-Glu was as effective as L-Asp in displacing L-Asp specifically bound to cerebellar membranes and that the Aspartate agonist NMDA was ineffective, together with the aspartate antagonist D∝AA.

KA (corresponding to a glutamate molecule restricted to an extended conformation) had no affinity for the Na^+ independent binding site on cortical membranes (this study). A similar result was obtained on the effectiveness of KA as a displacer of L-Asp bound to cerebellar membranes (Sharif and Roberts, 1981). The other differences in the structural requirements of the binding sites in cerebellum and cerebral cortex may reflect a genuine difference in the type of binding site found in these two brain areas. Those in the cerebellum may show a greater level of cross-reactivity than those in the cortex which may be more restrictive in their structural requirements - possibly arguing for a difference in the functional demands made on different receptor types, reflecting the complexity of input into the cortex.

The Na^+ dependent site at 50 days of age will bind L-Asp, L-Glu and D-Glu with high affinity, which is very much in keeping with other evidence that these three amino acids share the same transport site. The IC50 found by displacing either glutamate or aspartate from this site was the

same for all the effective ligands. D-Asp also displaces L-Asp bound to this Na^+ dependent site although its potency is 10 times weaker than for the other 3 molecules. Both the kinetic data and the pharmacological data support the view that this Na^+ dependent site may contribute to the removal of glutamate and aspartate from the synaptic cleft when their activity is terminated. The structural demands of this binding site are the same at 20 days as at 50 days, with the exception of its ability to bind D-Glu which only becomes capable of displacing aspartate bound to its Na^+ dependent site after the 20 day age point. This effect of D-Glu was also found using L-Glu as the displaceable ligand and is rather difficult to explain.

In contrast, the structural specificity of the Na^+ independent aspartate binding site changes dramatically during the period 20-50 days, paralleling the dramatic changes in the kinetic parameters of the binding over the same period. The affinity of this site for L-Asp is much lower at 20 days than at 50 days, the relevant IC50's being 7.2×10^{-7}M at 50 days and 5.0×10^{-6}M at 20 days. The interesting observation though is that L-Glu has an even greater ability than L-Asp to displace ^{3}H-L-Asp binding at this earlier age (IC50 2.0×10^{-6}M), in contrast to its ineffectiveness at displacing any of the binding at 50 days. This ability of L-Glu to displace L-Asp is reflected by kainic acid, a strict glutamate analogue that like glutamate has some affinity for the Na^+ independent aspartate site at 20 days, but not at 50 days.

The fact that the Na^+ independent L-Asp binding site is present at higher concentration and with a much lower affinity for its ligand at 20 days than at 50 days, could mean that L-Asp is reacting with other populations of receptors at 20 days as well as with its own binding sites. The fact that this binding is of relatively low affinity could reflect

the fact that these different binding sites can adapt to the occupation by ligands that differ from each other slightly in their shape. Because of this adaptive capacity the receptor component would not be fixed into any one rigid conformation, at least at its acceptor site, and rigidity of shape is probably a prerequisite for highly specific, high affinity interactions between a binding site and its designated ligand. This view, that the site binding aspartate is relatively unspecified at 20 days of age, is reflected in the fact that it also has high affinity for L-Glu and more especially the conformationally restricted glutamate agonist kainic acid. This is not the case at 50 days when many fewer sites are available for L-Asp to bind to. In addition these sites will no longer bind L-Glu or kainic acid, showing preference for the aspartate agaonist MNDA and its antagonist D∝AA.

Thus, the kinetic and pharmacological data on the binding of Aspartate to rat cortical membranes support and strengthen its candidature as a neurotransmitter. At the same time the hypothesis is upheld that binding sites destined to subserve amino acidergic neurotransmission are relatively unspecified for either one of their two possible ligands (glutamate and aspartate) at the time of initial contact between presynaptic elements, and that the postsynaptic cell only becomes committed to glutamatergic or aspertergic transmission (with the concommitant increase in specificity for one or the other) as specific pathways become activated. The ontogenetic profile of the Na^+ independent L-Asp binding site shows the high affinity and low capacity characteristic of the adult only from 30 days of age.

5.6 Effects of freezing

The binding studies reported in this thesis were carried out using freshly prepared tissue only. Thus animals were killed in the morning, brain samples prepared, binding assay carried out and samples processed for scintillation counting as a continuous operation. The drawback is that a relatively quick method of fractionation of brain tissue had to be employed, yielding a crude preparation of synaptic membranes (essentially a purified P_2 fraction); instead of pure synaptic membrane fractions that may be obtained following fractionation procedures taking 6-8 hours to perform. The heterogeneity of the membrane sample used in binding studies obviously raised the question of the exact brain compartment responsible for the binding. The use of pure synaptic membranes would have obviated some of these difficulties but would necessitate, because of the time involved, samples being frozen and stored before use in the binding assay.

There have been conflicting reports on the effects of freezing on glutamate binding. Biziere et al (1980) reported that binding was not affected by freezing membranes from many different brain areas. Similarly Vincent and McGeer (1980) reported no loss of Na^+ dependent binding in the striatum after membranes had been frozen and rethawed. In contrast there are reports that glutamate and aspartate binding to cerebellar membranes is severely decreased after freeze/thawing (Foster and Roberts, 1978; Sharif and Roberts, 1981).

Consequently, the effects of using frozen and thawed cortical membrane fractions in the glutamate and aspartate binding assays was examined.

Results

Initial studies were carried out using membranes prepared from adult (50 day old) rats and showed that there was a dramatic increase in the levels of Na^+ independent glutamate binding after freeze/thawing (Table 5.6). This substantial (x 9) increase represented a rise in the maximal number of binding sites available, from 6.4 pmols/mg protein in fresh tissue to 54 pmols/mg protein in previously frozen tissue. There was no change in the affinity of this Na^+ independent receptor for glutamate. In contrast Na^+ dependent binding of glutamate was decreased by about 50% in frozen/rethawed tissue. Na^+ independent aspartate binding was not affected by freezing whilst Na^+ dependent binding of aspartate, like that of glutamate, was decreased by 50-60%.

The dramatic difference in the effects of freezing between the Na^+ independent binding of aspartate and that of glutamate in the adult was strongly indicative of the existence of different binding sites for the two ligands. This prompted the question "Is this difference apparent in frozen membranes prepared from younger rats?"

The results of such an experiment can also be seen in Table 5.6 and shows that Na^+ independent glutamate binding is slightly decreased in freeze/thawed membranes prepared from 20 day old rats, and that Na^+ independent aspartate binding is effected in an identical way, i.e. a slight decrease in binding. The effects of freeze/thawing on the Na^+ dependent binding of both glutamate and aspartate in membranes prepared from these young rats is identical to that found in the adult, i.e. an approximately 60% decrease.

TABLE 56

The effects of freeze-thawing on the subsequent binding of ^{3}H-L-Glu and ^{3}H-L-Asp to cortical membranes prepared from rats at 50 and 20 days of age. The control values are the levels of binding to freshly prepared tissue. Freeze/thaw tissue was frozen for 24 hours as a suspension in 50mM KPO_4 buffer pH 7.1, and was used in the binding assay directly upon thawing. The results are mean ± SEM of 5 separate determinations.

Glutamate binding (B max: pmols/mg protein)

	Na^+ Independent		Na^+ dependent	
	Control	Exp	Control	Exp
50 dy	6.2 ± 0.57	54.0 ± 0.26	210 ± 19.2	108 ± 14.1
20 dy	8.47 ± 1.05	6.2 ± 2.1	82.4 ± 4.19	38.6 ± 2.8

Aspartate Binding

	Na^+ IND		Na^+ DEP	
	Control	Exp	Control	Exp
50 dy	19.4 ± 1.6	18.9 ± 1.4	361 ± 18.4	152 ± 16.0
20 dy	54.5 ± 2.8	48.2 ± 5.6	1157 ± 37.0	536 ± 42.0

Discussion

One obvious interpretation of the results is that they provide additional support for the hypothesis that glutamate and aspartate bind to the same Na^+ independent (postsynaptic?) receptor in younger animals. This being the case, their binding to such a receptor would be expected to be similarly affected by any modification procedure. However, by 50 days such receptors have become specified for either glutamate or aspartate and thus are now differentially affected by such procedures as freezing and subsequent thawing of membranes.

It is a little more difficult to explain the very large increase in Na^+ independent glutamate binding to previously frozen membranes prepared from adult rats. Rupturing of the membrane bilayer by freezing could expose many integral membrane protein components capable of binding glutamate. It is difficult to assess a physiological role for such hidden binding sites, but they may be involved in long term 'plastic' responses of the neuronal cell surface such as supersensitivity or long-term potentiation (LTP). Such a physiological role is implicated by the fact that these uncovered binding sites have the same Kd value ($0.27\mu M$) for glutamate as those exposed in fresh tissue. The latter phenomenon (LTP) has been shown to occur in the hippocampus following brief periods of high frequency stimulation (Dunwiddie and Lynch, 1978). Glutamate is strongly favoured, on the basis of neurophysiological and neurochemical evidence, as the transmitter utilized by several afferent and efferent pathways to and from the hippocampus (Storm – Mathisen, 1977). Baudry and Lynch (1980) have suggested that the physiological mechanism by which LTP occurs may be an uncovering of hidden receptors by a Ca^{++} activated protease. The phenomenon of LTP may be expected to be

much more pronounced in areas that are likely to possess a high degree of functional plasticity reflecting their involvement in brain functions such as learning and memory. As a corollary, brain areas that are essentially involved in the control of muscular coordination and therefore exhibit a much more invariant wiring pattern throughout their ordered structure may not be capable of L.T.P. If this is the case then it may explain the presence of hidden receptors in the cerebral cortex and hippocampus capable of exposure by freezing, and the absence of such receptors in the cerebellum where L.T.P. would not be expected.

The findings that Na^+ independent aspartate binding does not increase upon freezing adult membranes, whilst binding of glutamate does, could be a reflection of differing roles for the two amino acids in the mediation of synaptic plasticity, although other possibilities exist.

5.7 Localization studies - The Reeler Mutant Mouse

The binding studies reported here have utilized membranes prepared from the whole cortex cleared of underlying structures and white matter. The reported results have provided a useful insight into the types of interaction that exist between the amino acids and their receptors in this part of the brain. However, the cortex itself is heterogenous both in terms of cell type and of the known functional demands made on various cortical areas by an enormous diversity of afferent connections. A more accurate quantitative assessment, and an analysis of the factors modulating the binding may be best studied in a more localised cortical area receiving a rich glutamatergic projection. In an attempt to identify such an area, cleaned cortices were dissected into four sections by tangential cross section dividing the cortex roughly into visual cortex, motor cortex and two intermediate zones.

Although several different estimations of Na^+ dependent and Na^+ independent glutamate binding were carried out on each section (i.e. utilizing the cortices from several different animals) results were inconclusive. Variations in levels of binding to the same areas of individual rats was pronounced and statistical tests on pooled results showed no real differences in the levels of binding in these cortical areas. Several explanations for these disappointing experimental findings are plausible; glutamatergic neurons may project into all cortical regions and zone dissection may have to be extremely accurate, and coupled with a highly sensitive assay technique before major differences can be identified.

A promising line of investigation into the problem of localization of glutamatergic projections in the cerebral cortex was offered by a consideration of the abnormal brain structure of the autosomal recessive

mutant mouse 'Reeler.' 'Reeler' is alone amongst the neurologically abnormal mice, characterized by aberrations in cerebellar cytoarchitecture and wiring patterns, in that the reeler mutation also affects, detrimentally, the normal distribution of cell types in the cerebral cortex.

The normal cortex of mice is separated into distinct lamina formed by a segregation of classes of cells into tangential strata. The cells in each of the laminae have in common a distinct somatic and dendritic morphology and a characteristic pattern of axon deployment as their anatomical features. In addition, all the cells in any one lamina share the same pattern of afferent and efferent connections and are all orientated in a particular way relative to other neurons.

Comprehensive studies by Caviness and colleagues have established that these general attributes of cellular organisation are essentially preserved in the cortex of reeler mutants (Caviness, 1977; Caviness and Rakic, 1978) despite the fact that the relative location of the laminae are reversed in the reeler cortex compared to the normal cortex. During the process of morphogenesis in the normal and in the reeler cortex, cells at the earliest stages of cell proliferation, on embryonic day 11 (E11), migrate to the pial surface of the immature cortex guided by radial glial fibres (Rakic, 1972). In both normal and mutant mice migration is complete by E13. The next two days are characterized in the normal animal by two events. The first of these is the development of a cortical plate of cells delineated above and below by a cell sparse plexiform layer. Simultaneously the cells subdivide into two distinct populations. The larger population migrates deep into the cortex, whilst the much smaller population remains superficial, constituting the plexiform layer of the mature cortex (Caviness, 1977). These deeply directed cells differentiate into the 'polymorphous

and large pyramidal cell layers of the normal cortex. Cells subsequently arriving in the cortical plate region migrate to progressively more superficial layers to form a characteristic distribution of cell types.

The developmental events at E13 are dramatically different in the reeler, however, as the cortical plate cells do not become subdivided into separate populations, but remain together and occupy the most superficial layers of the cortex. This means that a plexiform layer containing few cells does not arise. The cells of the cortical plate in the reeler subsequently differentiate to give a superficial polymorphous lamina, and, immediately below, a strata of large pyramidal cells. Thus later generated cells are forced to occupy positions successively deep to these layers, essentially reversing the normal pattern of development. Delong and Sidman (1970) have hypothesized that the lack of division into sub-populations at E13, and the subsequent non-migration of predestined large pyramidal cells, may be due to an altered (increased) affinity of these cells for each other or for non-neural structures at the cortical periphery. This explanation presupposes that the role of the gene at the reeler locus may be to specify levels of cell surface components mediating recognition phenomena.

Despite this reversal in lamina structure, efferent projections from the cortex arise in a similar manner and innervate the same target zones as in the normal mouse. Axons terminating in the cortex from other brain regions, and constituting the main afferent cortical pathways, are also very similar in the normal and reeler mice in that they synapse with the same target cells in both animals.

By necessity these are alterations in the route followed by neural elements entering and leaving the cortex of reeler mutants, but

specificity of connections is maintained, on the whole, with remarkable accuracy.

There is however, one afferent system where abnormal connectivity is apparent. Axons from the olfactory bulb of normal and reeler mice travel in the lateral olfactory tract (LOT) and their terminals are then distributed densely to lie superficial in the pyriform cortex. From here, in the normal animal, they contact with the distal portion of the dominant apical dendrite arising from pyramidal cells immediately below, i.e. in the layers of smaller pyramidal cells. These cells, in contrast, lie in the deepest strata in the reeler and, although some of their apical dendrites may be radially aligned, they may be descending as well as ascending. In addition, many of these cells are tangentially aligned. This is also the case for the larger pyramidal cell types lying more superficially in the reeler cortex. These tangentially oriented or descending dendrites do not penetrate into the superficial cortical zone of terminals emerging from the L.O.T. Additionally, and in contrast to other afferent systems, the olfactory fibres do not penetrate deeper into the cortex to meet tangential dendrites of the small pyramidal cells. Thus the number of synaptic connections made by afferent LOT fibres in the cortex of reelers is considerably reduced, being restricted to the relatively small number of radially orientated ascending apical dendrites arising from deeply placed pyramidal cells.

In normal animals these terminals usually synapse on one dendritic spine where a strong, anatomically distinct connection is formed. In contrast, in the reeler, the terminals are much larger and may span three or four dendritic spines, although forming much looser, and therefore less stable, synapses (Caviness et al, 1977). This abnormal situation may also be characterised by transient synapses, i.e. the continual

regeneration and degeneration of weak synaptic connections with the equilibrium in favour of degeneration.

The acidic amino acids are strong candidates for the role of transmitter utilized by the cells of the olfactory bulb terminating in the pyriform cortex. This candidature is based on many types of evidence, but there does seem to be a species difference in the usage of either glutamate or aspartate. Thus, in the rat, LOT stimulation causes an increased release of aspartate only, in a Ca^{++} dependent manner (Collins, 1979). This amino acid is therefore suggested to be the prime transmitter of the afferent path into the olfactory cortex (Collins and Probett, 1981) although there is some indication that glutamate may be released at some of the L.O.T. fibre terminals (Harvey, et al, 1975; Collins, 1980; Collins et al., 1981). In contrast there is considerable evidence that glutamate, not aspartate, is the transmitter utilized by this pathway in the guinea pig. Electrophysiological studies have demonstrated the presence of nerve cells within the target area of the L.O.T. fibres that are particularly sensitive to glutamate (Richards, 1977). In addition, glutamate content of the piriform cortex is considerably reduced after olfactory bulb ablation (Bradford and Richards, 1976). Stimulation of the L.O.T. also causes an increased Ca^{++} dependent release of glutamate from the piriform cortex (Yamamato and Matsui, 1975; Bradford and Richards, 1976). Although there are no reports in the literature of such studies in the mouse brain, the similarity between the murine and rodent olfactory system would suggest that the amino acids may also be transmitter candidates in the species. If this hypothesis is correct, then it may be expected that binding sites for glutamate on cortical membranes prepared from reeler would be severely reduced in number

compared to those in normal animals. Although the pyramidal cells that normally receive the innervation are present they cannot form synapses with the incoming olfactory fibres. A common feature of the developing nervous system is that cells deprived of their normal afferent innervation will not develop receptors for the afferent transmitter or will 'internalize' receptors formed. Because this abnormality is restricted to the olfactory system as opposed to other afferent inputs any differences that were found in glutamatergic parameters would be strongly indicative of a transmitter role for glutamate in that system.

From these considerations membranes prepared for the cerebral cortices of reeler and normal mice were used to carry out binding studies. The results are shown in Table 57.

TABLE 5.7

Results Values given are mean ± SEM.

	Na^+ independent Binding (pmols/mg protein)	Na^+ dependent Binding (pmols/mg protein)
Normal mice	14.5 ± 1.2 (5) C	79.3 ± 4.6 (5) A
Reeler	11.25 ± 0.3 (6) D	75.9 ± 3.4 (6) B

n values in brackets.

Differences in means A and B non significant.

Differences in means C and D; $p < .025$.

The arguments put forward earlier in this section suggest that if glutamate is the transmitter of the olfactory pathway into the cortex, then levels of glutamate binding to membranes prepared from the cortices of the reeler mutant mouse may be expected to be considerably reduced. In the presence of Na^+, binding of glutamate is very similar in both normal and mutant mice. Although binding in the presence of Na^+ is usually associated with binding to a transport site involved in the active high affinity uptake of transmitter from the synaptic cleft, this uptake may also subserve other functions. Even in the absence of transmitter glutamate, an efficient uptake system may be necessary not only to accumulate the amino acid for metabolic purposes, but also to prevent extracellular levels of this potent excitatory amino acid reaching thresholds evoking polarization. Such a function may be carried out by a high capacity low affinity transport system in addition to the low capacityhigh affinity system, that may also function in glutamatergic transmission. Loss of binding sites within this second system may therefore be masked by the much greater number of low affinity sites, especially when binding is measured at a single concentration of

ligand as is the case here. A loss of binding sites might only be detected following a detailed study of binding kinetics in the presence of Na^+ utilizing a wide range of ligand concentrations.

On the other hand, Na^+ independent binding is significantly reduced in the mutant compared to the normal mouse ($p<0.025$), corresponding to a 22.5% reduction. However, from a consideration of the drastic reduction in synaptic connections in the olfactory cortex then a much greater drop in levels of Na^+ independent binding might be expected if glutamate is indeed the transmitter of this afferent system. There are of course many possible explanations for this finding. First of all, glutamate may be utilized as a transmitter by many of the afferent pathways into the cortex and for communication between different cortical areas. In this case, loss of synapses in one system only would not make such a large difference in overall levels of binding. This is quite in keeping with a ubiquitous role for the amino acid as excitatory transmitter in the CNS. It could also be that glutamate may exert an excitatory effect by a synergistic action with the putative excitatory transmitter aspartate (Collins, 1976). A similar analysis of the differences in aspartate binding between reeler and normal mice would then produce a similar result. The additive loss of glutamate and aspartate binding sites in reeler may represent a very large decrease compared to the number of glutamate and aspartate binding sites in the normal animal.

A further possibility is that loss of synaptic connections and therefore degeneration of presumptive receptor complexes in olfactory cortex may initially be considerable in the reeler. The response to this situation, however, may be a marked increase in receptor number at the small number of synapses that do form, or an increased affinity of receptors for this lignad. This evoked supersensitivity of some cortical

neurons for the olfactory transmitter could partially compensate for the loss of receptor capacity in the majority of normally receptive cells leading to a decrease in levels of binding only of the proportions found in this study.

Although these results are not as conclusive as were hoped, they do raise many possibilities for future studies and establish the reeler as an important experimental tool in the search for specific amino acidergic pathways. The use of congenital mutants in this type of work is especially attractive as it avoids the need to manufacture disruption in cortical synaptology either by mechanical or chemical lesioning techniques, or by the use of sensory deprivation. Any of these methods may produce changes in neurochemical parameters that are primarily trauma-related as well as raising ethical problems relating to animal experimentation. The results do not contradict the view that glutamate may be the transmitter of the cortical afferents carried in the L.O.T. of the mouse.

CHAPTER 6

GENERAL DISCUSSION

6.1 CRITIQUE OF BINDING DATA

The experiments reported in this thesis were initially carried out to test the hypothesis that the acidic amino acids glutamate and aspartate are neurotransmitters utilized by afferent pathways to the cerebral cortex and perhaps for communication between different cortical areas. If the amino acids are transmitters they must fulful accepted criteria for neurotransmitter status. One of these criteria is that receptive neurons must possess high affinity, low capacity receptor sites for the putative transmitter, as it is almost certainly through the interaction with such membrane bound receptors that transmitters exert their physiological effect, evoking changes in the postsynaptic cell that lead to a shift in transmembrane potential.

I have demonstrated that such sites exist for both glutamate and aspartate on cortical membranes prepared from adult rats (Section 5.1 and 5.5). The binding to these sites is not dependent on the

presence of sodium and therefore cannot represent binding to transport sites that have a strict requirement for this ion.

Post synaptic receptor sites, from theoretical considerations, should show high affinity for their ligands, to enable them to respond to very small changes in the concentration of the transmitter in the synaptic cleft. The affinity constants for the binding of glutamate and aspartate to their Na^+ independent sites are very similar at around 0.3μM. This is very much in keeping with Kd values found by many other workers, and, in addition, the reported affinity constants show remarkable agreement between different brain areas. The crucial consideration here then is not that there are binding sites for glutamate in the brain, but whether or not these binding sites fulfill the criterion of high affinity. A major problem here is that any definition of 'high affinity' must be either arbitrary and subjective (depending on whether one believes glutamate to be a neurotransmitter or not!) or must have a standard of comparison.

The affinity constants commonly reported for the interaction between glutamate and its two transport sites in brain tissue are about 2-5μM and 50μM respectively. Compared to these values a Kd of 0.3μM certainly represents a dramatic increase in affinity of at least 10 fold. This difference in affinity between glutamate binding sites in the brain must represent different physiological functions. As the Na^+ independent sites do seem to be concentrated in the synaptic membrane fraction of brain tissue (Section 4g) it is quite conceivable that they could be subserving a function in neurotransmission, with these "high affinity" sites synonymous with the postsynaptic receptor.

However, if the Kd of this site is compared to the affinity constant

of the receptor sites for the classic transmitters acetylcholine and GABA, then the concept of high affinity becomes a little clouded. The nicotonic receptor for Ach has a Kd of 4nM and the GABA receptor of roughly 50 times higher affinity than the glutamate or aspartate high affinity site.

In the introductory chapter of this thesis, I argued that it may be misguided and therefore uninformative to apply criteria of neurotransmission based on the mechanism of action of Ach, to the search for new transmitter molecules. This is especially true when the acidic amino acids are considered, because of their importance as metabolites in the cell, being most intimately involved in protein synthesis and in the control and regulation of nitrogen metabolism. These amino acids are ubiquitously present in the body and, although they do not easily cross the blood/brain barrier, they are nevertheless present in the C.S.F. in high concentrations. Although estimates of this concentration vary considerably, an average free glutamate concentration in CSF of 21mM has been arrived at (Johnston, 1978; McGabe et al., 1977). Electrophysiological studies have shown that iontophoretically applied glutamate can depolarize CNS neurons at concentrations of 10^{-5} or 10^{-6}M <u>in vivo</u> (Curtis, 1965) or <u>in vitro</u> (Hosli et al., 1979).

Obviously, post-synaptic receptors triggering intracellular events leading to membrane depolarization should not be responsive to levels of excitatory ligand normally occurring in the extracellular space, or more particular in the synaptic cleft where the receptors occur. A low affinity constant for glutamate receptors in the range of the Ach receptor would result in continual high levels of activation at all times, which could lead to cell death via the excitotoxic mechanism proposed by Olney (1975) and mentioned earlier. A lower affinity constant

then, far from being an argument against glutamate being a transmitter, would be an absolute requisite if glutamate were fulfilling such a role. The same situation applies to the role of aspartate as neurotransmitter, with an estimated CSF concentration of 6.6mM.

As already discussed in Chapter 2, the kinetic parameters describing the in vitro binding must at least be compatible with the dose dependancy of the electrophysiological effect. The estimated Kd values for glutamate and Aspartate for the Na^+ independent sites are approx. 0.3μM - of the same order as the concentrations needed to evoke EPSP's. Discrepancies between exact values can not only be easily explained, but also expected from a consideration of the unknown contributions of such factors as spare receptors and different receptor sites (discussed under receptor theory Chapter 2) to the in vivo results.

Nevertheless, despite this relatively low affinity for glutamate, minimal levels of this amino acid needed to evoke EPSP's are of the same order (if not lower) as the concentration of glutamate normally circulating in the C.S.F. This suggests the possibility that glutamate continually exerts a depolarizing influence on receptor neurons so that the EM of the membrane is shifted considerably towards zero and maintained at a value just sub-threshold for depolarization. In these circumstances, as pointed out by Johnson (1978), the post-synaptic cell could be extremely responsive to very small increases in excitatory transmitter in the synaptic cleft and released from the presynaptic nerve ending in response to the arrival of a nerve impulse. If this is indeed the case, it raises questions as to the physiological significance of release studies that show large changes in the concentration of extracellular amino acids after stimulation of the presynaptic input. It is also possible of course that this depolarizing influence of glutamate is its

only contribution to synaptic function and it therefore potentiates or modulates the activity of an as yet unidentified excitatory transmitter.

Pertinent to this line of thought is the question of whether or not the binding sites being measured in <u>in vitro</u> studies have a solely synaptic location. My own studies, and those by other workers (Cotman et al, 1981), demonstrate a dramatic increase in the concentration of Na^+ independent binding sites in a purified synaptic membrane preparation. However, iontophoretic studies demonstrate conclusively that perikaryal membranes are highly responsive to applied glutamate and aspartate although the synaptic excitatory receptors are thought to be dendritically located. Further purification of this synaptic plasma membrane fraction to give a preparation enriched in synaptic junctions yields a recovery of only 11% and 26% of the glutamate and aspartate receptors respectively compared to their levels in SPM's. In addition, Na^+ independent binding sites are easily demonstrable at high levels in a P_2 fraction suggesting some extrasynaptic location. Interestingly, the kinetic constants defining the binding are the same, whether a P_2 fraction or a purified synaptic membrane fraction is used, suggestive of a similarity of function. Again, the preceeding discussion is equally applicable to aspartate.

So there is evidence that physiologically relevant receptors for glutamate exist both at the synapse and at much more widespread extrasynaptic locations, most probably on the perikaryal membrane. The fact that extracellular levels of the amino acids are sufficient (without evoked neuronal activity) to maintain a EM considerably shifted towards the depolarization threshold suggests a role for the Na^+ dependent binding.

There is very strong evidence that such binding is to an uptake site that is predominantly located on glial cell membranes (Henn, 1976; Hertz, 1979). My own studies on the subcellular location of the binding show that whilst there is an 8-fold purification of Na^+ independent binding sites in the synaptic membrane fraction there is only a $1\frac{1}{2}$ times purification of the putative uptake sites. Although this could be a reflection of the fact that transport sites for the rapid uptake of glutamate could be present to some extent on synaptic membranes it could also be explained by their presence in increased concentrations on membranes from glial cells surrounding the synapse. The preparation of crude synaptic membranes yields samples that are contaminated with membranes from cell types intimately associated with the synaptic region in vivo. Very recently the compound 4-acetamino-4-isothiocyano-2,2 disulphonic acid stilbene (SITS) has been suggested as a specific glial inhibitor of glutamate uptake (Waniewski and Martin, 1983). The use of this compound in binding studies may also inhibit the glial component of the binding, although its mechanism of action has not been fully clarified.

Nevertheless, the fact that the purification factor is so low when comparing a P_2 to a SM fraction indicate that a vast proportion of the sites are not associated with the synapse. The function of these extrasynaptic high affinity uptake sites could, then, be two-fold. They could maintain normal levels of the excitatory amino acids within narrowly defined limits, acting as a fine control of the modulatory effect of the amino acids in eliciting shifts in E.M. towards the depolarization threshold. In addition, high levels of glial uptake at elevated C.S.F. concentrations could be a vital protection mechanism against the excitotoxic effects of these amino acids.

It may be that this 'fine tuning' of the synaptic levels of glutamate and aspartate by the high affinity transport mechanism is the only role for such systems.

Uptake studies show many anomalies. Although many workers have maintained that a high affinity transport mechanism for Glycine occurs in the spinal cord where it is a transmitter, but not in the cerebral cortex where it is not thought to have a transmitter function, (Logan and Snyder 1972) there has been one report that questions this. Using synaptosomal fractions of rat cerebral cortex Peterson and Raghupathy (1973) reported a transport process for Gly with a KM of 2.5×10^{-5}M. Later, the same authors reported the presence of high affinity transport mechanism showing strict Na^+ dependency for the non-transmitter amino acids serine and threonine in brain cortical synaptosomes from newborn rats (Peterson N.A. and Raghupathy E. 1978). Other workers too have found evidence of high affinity transport of non-transmitter amino acid. Thus high affinity mechanisms exist for arginine, serine, leucine, tryptophan and Tyrosine in purified synaptosomes prepared from cerebral cortex (Balcar and Johnston 1974). Although these transport systems were not dependent on sodium the high affinity transport of proline was. Balcar and Johnston (1975) also reported the Na+ dependent high affinity transport of glutamine into rat brain slices.

The transport of the inhibitory amino acid G.A.B.A. by both high and low affinity systems is well documented in many brain regions. However a similar high affinity process for the accumulation of G.A.B.A. has also been demonstrated in rat sympathetic ganglia that contain very little G.A.B.A. or its related metabolizing enzymes (Bowery and Brown 1972). Similarly, as previously mentioned, Roberts and Keen (1974)

have shown that a high affinity transport mechanism exists for glutamate in the dorsal root ganglion, where it is not a transmitter. Thus it may be dangerous to assume that demonstration of a Na^+ dependent high affinity binding site in a particular region is evidence of a transmitter role for the endogenous ligands of such sites. Blockers of the high affinity transport site, such as Threo-3 hydroxy-aspartate and dihydrokainic acid, do seem to enhance responses to L-glu and L-asp applied in vitro, but not to NMDA or kainic acid which are not transported (Johnston et al 1980; Lodge et al 1980). In addition, however, is the fact that histidine, which competes only for low affinity transport sites, is also able to prolong L-Glu and L-Asp responses (Lodge, 1976). D-Glu and L-Homocysteate are excitatory amino acids that have the same onset and cessation of action as L-Glu and L-Asp even though they are removed only by a low affinity system (Cox et al., 1977).

So differential distribution of these transport systems (Na^+ dependent high affinity) may simply reflect differences in susceptibility of neurons to the excitotoxic effects of the putative transmitters. Alternatively, these high affinity systems could be a reflection of the important metabolic function of the amino acids. TRYP and TYR in particular are precursors for the biogenic amine transmitters. Very recently an endogenous peptide with high affinity for brain glutamate receptors has been reported (Zaczek et al 1983). This is N-acetylaspartylglutamate and if it is subserving a synaptic function glutamate and aspartate accumulated by their high affinity active transport systems may be serving as precursors.

In addition, interpretation of data on the uptake of L-Glu and L-Asp

is complicated by factors such as the extent of metabolism of the accumulated amino acid, although this can be overcome by using low temperatures or short incubation times. A more serious consideration is the extent to which exogenous amino acids exchange with endogenous pools. It has been shown that if synaptosomes or tissue slices are pre-labelled with ^{3}H-L-Glu addition of cold Glu (at concentrations in the range of high affinity uptake) will increase ^{3}H-Glu release in a saturable, concentration dependent manner (Levi et al 1976, Jones and McIlwain 1971). As stated by De Feudis (1975), this homoexchange process (of labelled amino acid for cold endogenous stored) could explain double affinity systems, in that at low concentrations (10^{-5}M) of added amino acid the stimulation of an exchange mechanism would lead to a significant increase in the amount of labelled amino acid entering which would be observed as a high affinity system. So net accumulation of amino acid is actually the sum of three processes; uptake, exit and exchange, all of which must be quantified. Only in the case of Glycine has net uptake of amino acid been demonstrated at low concentrations of substrate (Cox and Bradford 1978). All other reports of net uptake can be accounted for by low affinity systems (Cox and Bradford 1978).

Heteroexchange between endogenous and exogenous pools of different acidic amino acids may also occur. Levi et al (1976) showed that low concentrations of unlabelled glutamate stimulated synaptosomal release of endogenous labelled aspartate. If these high affinity mechanisms are indeed predominantly exchange processes then they are unlikely to be involved in the inactivation of released transmitter. The ubiquitous low affinity transport systems may be the major influence in modifying responses to excitatory amino acids (Cotman et al 1981).

6.2 Distinct Glutamate and Aspartate Receptors?

Electrophysiological studies have demonstrated very little difference in either the excitatory potency or the physiological response characteristics of the two dicarboxylic amino acids. Iontophoretic application of either glutamate or aspartate to feline motorneurons, for example, gave depolarizations with fast onset and recovery, accompanied by a small increase in membrane conductance (Lambert et al, 1981). Both are equally strong claimants for a role as central excitatory neurotransmitters. The data reported in this thesis show that binding sites exhibiting kinetic parameters compatible with neurotransmission exist for both amino acids. The question that arises then is, are the binding sites indicative of physiologically distinct roles for the two amino acids, do they mediate transmission into the cortex from different afferent pathways? Alternatively is their action synergistic? Are they both released from the same nerve endings to interact with the same receptors, their subsequent effect on ion movements across the post synaptic cell membrane being additive? It is also possible that only one of the amino acids is utilized *in vivo* as a transmitter and that *in vitro* electrophysiological and binding studies demonstrate only that one is an agonist of the other.

Such questions are not merely academic. If both compounds are transmitters utilized by different pathways this has important implications for trying to pinpoint the malfunction in pathological conditions and in the design of specific therapy. Pharmacological agents must (or should) only be effective where required!

A precedent for the hypothesis that glutamate and aspartate may work synergistically is found at the crustacean NMJ, where there is

considerable evidence to show that glutamate and aspartate are released together upon electrical stimulation of the presynaptic nerve (Freeman et al, 1981). Convincing experiments show that when L-Glu and L-Asp are applied simultaneously to enuromuscular preparations of the lobster, the dose response curve is shifted to the left, although no effect on the maximum response elicited by glutamate is seen (Shank et al, 1975). The application of L-Asp alone did not exert a depolarizing action on the postsynaptic cell even at concentrations 10 times higher than those potentiating the glutamate effect (Freeman et al, 1981). Even in preparations where aspartate alone does cause EPSP's, the postsynaptic response to simultaneous application of glutamate and aspartate is greater than the sum of the responses when each amino acid is applied alone (McBurney and Crawford, 1979). The only argument concerns the mechanism by which this potentiation is brought about. One group, working with lobster Homarus Americanus, favour the explanation that aspartate activates allosteric binding sites on the postsynaptic receptors, which induces an increase in the affinity between glutamate and its binding sites on the same receptors (Freeman et al, 1981). McBurney and Crawford (1979) have evidence from crab Maia squinado, that seems more compatible with the explanation that aspartate exerts its potentiating effect by inhibiting glutamate uptake causing a prolongation of glutamate evoked synaptic currents. So is it possible that in the mammalian CNS too, glutamate and aspartate may be released together and may function as a transmitter/modulator pair?

It may of course be impossible to give a simple 'yes or no' answer, in that the putative transmitters may well work synergistically in a particular brain area whilst the innervation to a different brain area

may be solely glutamatergic or aspartergic. Although there have been many studies of the electrophysiological effects of microiontophoretic application of the amino acids and their analogues in the CNS (Krnjevic and Phillis, 1963; Curtis and Watkins, 1963; Curtis, 1965; Duggan 1974; Hicks and McLennan, 1979), there has been no report of synergism. However, although virtually all cells respond to administration of glutamate or aspartate showing very similar effects on membrane conductance and firing rate, it is possible in some areas to demonstrate differences in the levels of the amino acids needed to evoke the response (Duggan, 1974; Hicks and McLennan, 1979). This would suggest that, although linked to the same ion channels, there are far fewer receptors for one of the amino acids than for the other. Alternatively, the receptors may be specific for the more active of the putative transmitters, the other acting as a partial agonist at these specific sites and thus evoking the same postsynaptic response only at much higher levels. This thesis has been particularly concerned with the nature of the transmitter released from afferents of the cerebral cortex. The kinetic parameters describing the binding of glutamate and aspartate to cortical membranes do not in themselves offer any insight into the problem of whether or not the two amino acids are binding to the same receptor. The affinity constants for the binding of both ligands are almost identical. The density of the binding sites is greater for aspartate than for glutamate, however, and this could indicate that different populations of receptors are being measured.

The displacement studies summarized in Tables 53 and 55 were carried out to distinguish between glutamate and aspartate binding sites on the basis of their pharm c logical requirements. From a considera-

tion of these results it is possible to distinguish between the Na^+ independent binding sites for glutamate and aspartate on adult rat brain membranes. Aspartate bound to its high affinity (Na^+ independent site can only be displaced by itself and by the aspartate analogues NMDA and DL ∝ AA and not by glutamate analogues G.D.E.E. or K.A. Reciprocally, L-Glu cannot be displaced from its binding site by L-Asp or aspartate-like analogues.

N.M.D.A., together with kainic acid and quisqualic acid, is one of the most potent excitatory amino acids. In the frog spinal cord a close parallelism has been demonstrated between the relative abilities of amino acid antagonists of the mono- and di-amino dicarboxylic acid series (characterized by D∝AA) to antagonize NMDA-induced responses and their relative potencies as depressants of dorsal root evoked synaptic excitation. Thus it seems very likely that NMDA is activating receptors that are physiologically activated by an excitatory amino acid. In addition, there is considerable evidence that is suggestive of these NMDA receptors being aspartate preferring (Watkins, 1981). Investigations of the structural features of agonists acting on NMDA receptors have suggested a 3 point attachment site with spatial parameters (separation of carboxyl groups) that could, however, accommodate either aspartate or glutamate. Until recently D aminoadipate (D∝AA) offered the best choice as an antagonist at these intermediate receptors. Recent reports, however, have shown that the ω-phosphono-analogue of D∝aminoadipate, (2, amino-phosphonovalerate, D-APV) is over 100 times more potent than D∝AA (Watkins, 1981; Olney et al, 1981). Very recently binding sites on rat brain membranes have been labelled with ^{3}H-D-APV (Olverman et al 1984). D-APV binding sites were present in all

areas of the CNS tested but particularly high in the hippocampus and cerebral cortex. The kinetic parameters describing the binding to cortical membranes show KD and BMAX values of 0.47μM and 4.1 pmols/mg protein respectively. These values are very similar to the KD and B MAX values for the binding of ^{3}H-L-Glu and ^{3}H-L-Asp to adult cerebral cortical membranes. The authors suggested on the basis of displacement studies with antagonists that ^{3}H-D-APV binds to the NMDA - type receptor identified in electro-physiological studies, and indeed of the three amino acids considered to be selective receptor agonists, NMDA was 25 and 80 times more potent than Quis and KA respectively at inhibiting the binding. However, L-Glu was as potent an inhibitor of ^{3}H-D-APV binding as APV itself and more than 10x as potent as NMDA or L-Asp. This study concludes that L-glutamate must be considered a strong candidate for the transmitter acting at NMDA receptors as well as Quisqualate receptors!

Although the work reported in this thesis supports earlier views that NMDA receptors are separate from glutamate receptors (see e.g. Watkins 1981 McLennan 1981) in that NMDA was totally ineffective at displacing glutamate bound to its Na^{+} independent site, there are other anomalies. Despite the fact that NMDA has a much greater excitatory potency than L-Asp it was approximately 10 times less effective than L-Asp in displacing ^{3}H-L-Asp from its putative post synaptic receptor site on cortical membranes. A direct characterization of NMDA binding sites using radiolabelled NMDA and therefore a knowledge of the kinetic parameters describing the interaction between this ligand and its receptor would be useful. However, although there has been one report of binding studies using NMDA and cerebellar membranes from mouse,

(Snodgrass, 1981), Roberts and co-workers were unable to repeat this work (Sharif, personal communication).

It is interesting to note that NMDA was also ineffective in inhibiting L-Asp binding to cerebellar membranes (Sharif and Roberts, 1981) and these workers also suggested that NMDA may not interact directly with aspartate recognition sites in this brain region.

A further explanation is given by the view that the interpretation of in vivo responses to L-Asp and NMDA are complicated by the fact that L-Asp is avidly taken up by high affinity transport mechanisms into brain cells surrounding the synapse, whilst NMDA is not transported. This would lead to a considerably reduced concentration of L-Asp at the synapse whilst the concentration of NMDA may not differ from that applied. Thus the relative differences in potencies may simply be a reflection of the differences in concentrations of ligands reaching the synapse.

Thus the usefulness of NMDA as an aspartate-preferring agonist in the brain as opposed to the spinal cord must therefore be questioned.

The antagonist G.D.E.E. did not have any effect on either L-Asp or L-Glu binding and therefore could not be used as a discriminatory tool. Studies using this antagonist as a displacer of binding may in anyway be complicated by the fact that high concentrations of G.D.E.E. exerts local anaesthetic effects on membranes (Roberts personal communication). In addition many commercial preparations of G.D.E.E. have been shown to be contaminated with Glutamate (Roberts, personal communication).

Other results reported in this thesis, however, are highly supportive of the view that glutamate and aspartate do indeed interact with

distinct binding sites. If the binding assays are carried out using membranes that have previously been frozen and thawed then there is an 8 - fold increase in the amount of binding (pmoles bound/mg protein) of L-Glu measured at a single concentration, whilst levels of binding of L-Asp to its Na^+ independant site are unaffected. This would seem to be totally incompatible with the proposal that glutamate and aspartate are binding to the same receptor complex.

The recent identification of glutamate binding sites that are Ca^{2+}/Cl^- dependent and sensitive to inhibition by the phosphonic acid derivative of glutamate L-2-amino - 4 phosphonobutyrate (L-APB) (Fagg et al 1982) may also be relevant to these results. It has been shown that this population of receptors are abolished by freezing whilst the smaller proportion of Ca^{2+}/Cl^- independent sites are not affected by freeze-thawing (Fagg et al.,1983). The binding studies reported in this thesis were carried out in a medium buffered by potassium phosphate so that the binding sites measured were probably of the Ca^{2+}/Cl^- independent type, which do not seem to be affected by freezing procedures. However it is still difficult to explain why there should be an increase in these sites on freeze/thawed membranes.

In addition, the developmental profile of the Na^+ independant binding sites are different for the two ligands. Binding sites for glutamate exhibiting a low site density (6.2 pmols/mg protein) and a high affinity (kd 1.32μM) are present at 20 days of age. This affinity constant for L-Glu increases to an adult value of 0.37μ M, although the maximal number of binding sites remains similar over this period. If aspartate is binding to the same receptor then it might be expected that the Bmax in young animals would also be similar to adult

levels. This is not the case. The binding of L-Asp to cortical membranes prepared from 20 day old rats is in fact highly anomalous, exhibiting a high density of 54.5 pmols/mg protein and a low affinity Kd 6.9 μM, which is difficult to equate with a physiologically significant post-synaptic receptor. The high affinity site Kd 0.3 μM can only be detected from 30 days of age, albeit at a lower site density than in the adult. The Bmax increases gradually over a time period when the density of glutamate binding sites remains constant. This suggests that the ability to specifically bind either glutamate or aspartate resides in different membrane components.

Nevertheless, these observations alone are inconclusive. As stated in C2 of this thesis, knowledge of the physiological significance of receptors identified through binding studies can only be arrived at following exhaustive studies linking these <u>in vitro</u> experiments with <u>in vivo</u> responses to the amino acids. Very recently, discriminatory agents have been discovered or manufactured that seem to specifically mimic or alter electrophysiological responses to one of the amino acids, whilst having no effect on responses to the other. The way forward in determining the separate transmitter roles for the dicarboxylic amino acids, together with their function throughout ontogeny, would be to use these agents to identify isolate and characterize the binding sites at various stages in development. This approach will be discussed in the final chapter of this thesis.

CHAPTER 7

THE WAY AHEAD

In vitro binding assays of the kind reported in this thesis have, I believe, provided an invaluable contribution to the problem of clarifying the kinds of interaction that occur between putative neurotransmitters and their recognition sites on brain cell membranes. I have shown that the dicarboxylic amino acids glutamate and aspartate bind to recognition sites that, on the basis of qualitative and quantitative criteria, are at least compatible with a role for them as neurotransmitters in the cerebral cortex. In support of this view, the developmental profile of these binding sites can at least be tentatively explained both in terms of the known morphological development of the brain and in terms of hypotheses that attempt to understand and explain the events that lead to specification of functional synapses to particular modes of neurotransmission.

Many problems remain, however, some actually generated by binding studies. In particular, there are many contradictions between information gleaned from in vitro studies and those obtained during neurophysiological investigations in vivo.

There are many contradictions in the literature concerning the discriminatory abilities of pharmacological agents acting at amino acid receptors (See e.g. McLennan 1983).

Nevertheless, it is essential to probe the structural requirements of excitatory amino acid receptors in the cortex for many reasons, not the least being because it is the first step in the design of specific therapy for brain malfunction. In addition, such studies may provide probes for investigating the different mechanisms of activation of the physiological response. As far as my own work is concerned, analogues with extreme specificity for the various types of amino acid receptors would be invaluable tools for the study of the development of transmitter systems in the brain. Any continuation of this work would have the pharmacological characterization of binding sites throughout ontogeny as its central theme.

Although aspartate and glutamate are relatively specific for their own Na^+ independent binding sites in adult rats (see Chapter 5), the situation is much more complex in the developing rat where a large degree of cross-reactivity between amino acid binding sites is apparent, making identification of the various components of the binding difficult, to say the least. Even in the adult, it is not easy to determine the exact configuration (either folded or extended) with which the amino acids interact with their receptors.

What then are the analogues that are likely to be of use in further studies probing the pharmacological requirements of amino acid receptors? Although the antagonist glutamate di-ethyl ester (G.D.E.E.) did not have any affinity for the glutamate binding sites reported in this thesis, this may simply imply that at the age points studied, glutamate may be

reacting with its receptors in an extended or partially extended configuration. G.D.E.E. may still be useful in identifying binding sites throughout development that can accept glutamate in a fully folded form (minimum separation of carboxyl groups 0.2nm). GDEE is especially potent at antagonizing the responses to the excitatory amino acid AMPA (α - amino - 3 - hydroxy-5 - methyl - isoxazole - 4 - proprionic acid) which is a glutamate analogue restricted in its separation of carboxyl carbon atoms to the minimum (0.2nm) by the steric interference from the methyl group on the 5 position of the isoxazole ring (McLennan 1981), see Fig. 7_1. Thus the simultaneous use of these two compounds (AMPA and GDEE) would provide a useful marker of amino acid receptors in the folded configuration. Recently, a report of the binding of ^{3}H-AMPA to brain membranes (Honore et al, 1982) shows that AMPA binding sites have no affinity for compounds known to interact with NMDA receptors in vivo. Curiously though, G.D.E.E. which is a potent antagonist of AMPA induced excitation in vivo did not inhibit the binding, which could imply that there are different antagonist and agonist states for the receptor.

Again, from a consideration of the separation distances of the carboxyl groups, the glutamate analogue and naturally occurring amino acid, ibotenate, may prove a very useful marker for binding sites accepting glutamate in a fully extended form, the degree of flexibility varying only between 0.46nm and 0.42 nm (Fig. 7_1). As aspartate can only extend to 0.38nm, aspartate receptors will not be able to bind ibotenate whilst the glutamate molecule can easily extend to the same maximum as ibotenate.

More recently, studies of the cyclic amino acid analogues, 2, 3 and

Fig. 7_1

Structural diagrams of amino acid analogues.

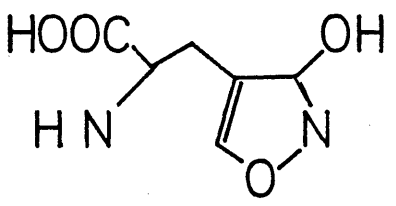
AMPA : Amino-3 hydroxy-5 methyl isoxazole-4 proprionate.

Ibo : Ibotenic acid.

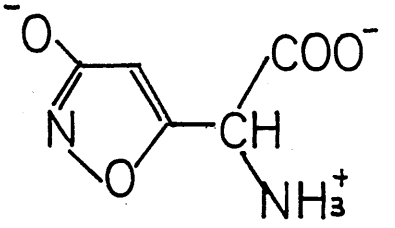
PDA : Piperidine dicarboxylic acid.

PZDA : Piperizine dicarboxylic acid.

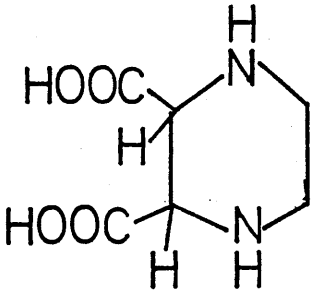
FIG 7₁



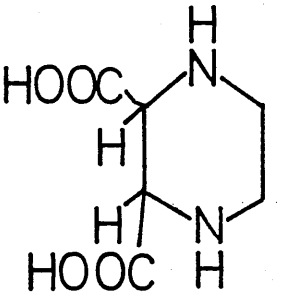
AMPA



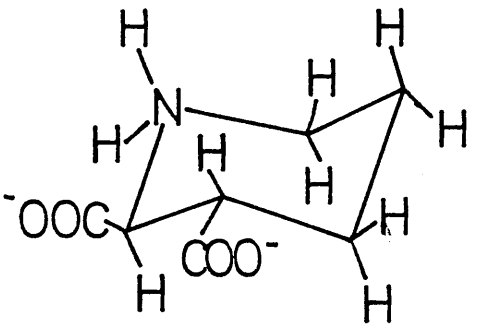
IBO



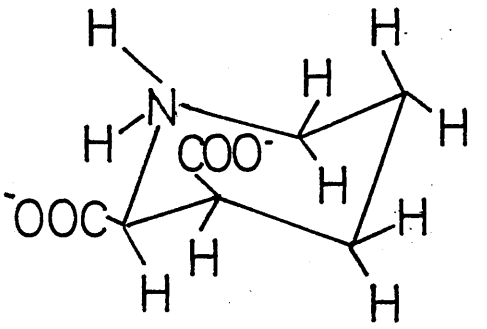
CIS 2,3 PZDA



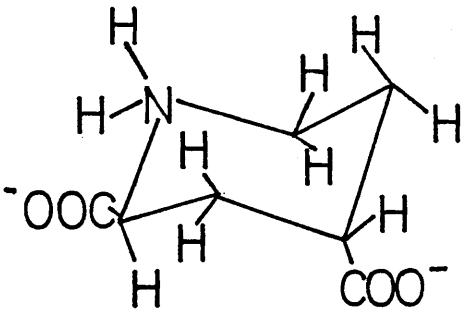
TRANS 2,3 PZDA



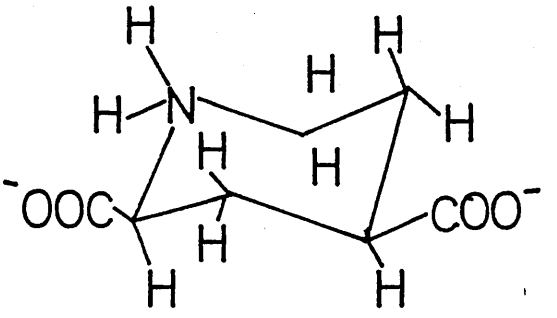
TRANS 2,3 PDA



CIS 2,3 PDA



TRANS 2,4 PDA



CIS 2,4 PDA

2,4 piperidine and piperazine dicarboxylic acids have suggested that they may be important tools in the characterization of amino acid receptors. Although two of these compounds, cis 2, 3 piperidine dicarboxylate (cis 2, 3 PDA) and cis 2, 3 piperazine dicarboxylate (cis 2, 3 PZDA), were antagonists of NMDA induced responses, and two others (the trans forms of 2, 3 and 2, 4 PDA) had NMDA - like agonist activity, the corresponding glutamate analogue, cis 2, 4 PDA, was inactive at the NMDA receptor (Watkins, 1981). As this compound also corresponds to glutamate in an extended position, it may prove to be a very specific marker for receptors accepting the fully extended glutamate molecule. All of these compounds are shown in Fig. 7$_1$.

The choice of pharmacological agents to probe the intermediate forms of the amino acid binding sites (and possibly corresponding to aspartate preferring receptors) is much wider. NMDA and trans 2, 3 PDA are both potent aspartate-like agonists. Trans 2, 4 PDA and amino dicarboxy-cyclopentate (ADCP), although glutamate-like analogues, correspond to a glutamate molecule in its partially folded form and are again potent agonists. Cis-ADCP is in fact pharmacologically indistinguishable from NMDA (Hicks et al.,1978) and is a more powerful excitant of neurones than is glutamate (Hall et al.,1979). The compounds cis,α-carboxycyclo-propylglycine (cis CPG) and 3 aminoglutarate may also be useful here. Although these compounds do have some affinity for Quis receptor types they have a strong preference for NMDA receptors (McLennan,1983). Both molecules (cis CPG and 3 aminoglutarate) are indistinguishable from aspartate (McLennan et al.,1982) pharmacologically. Discrimination between the relatively extended NMDA receptor type and the folded Quis receptor may also be possible by use of the antagonist Trans 5,

aminohex, -2-enedioic acid (AHED). This compound is structurally similar to Dα AA but has a double bond between its γ and δ carbon atoms. It is exclusively an NMDA antagonist (McLennan,1982) with an identical pattern of action to D α AA (McLennan et al.,1982). However unlike D α AA it is conformationally restricted by its double bond in an extended configuration. Recently, the most extensively studied group of amino acid analogues are those in which the ω carboxylate group has been replaced by a phosphonic acid moiety. These compounds have both agonist and antagonist properties, the latter of which is perhaps best characterized by 2-amino-5-phosphonovalerate (APV) which is a potent antagonist at the NMDA receptor. There is considerable evidence to show that the longer chain phosphono-derivatives, APV and 2-amino-5-phosphonoheptonate (APH), are very different in their effects to the shorter chain derivatives, in particular 2 amino 4-phosphonobutyrate (APB). APB (which is the derivative of glutamic acid) was ineffective as a displacer of APV bound to receptor sites on brain membranes (Olverton et al.,1984) whilst APV and APH were equipotent. Roberts et al. (1982) showed that APB was an effective inhibitor of glutamate binding to cerebellar membranes although it did not inhibit aspartate binding.

More significantly APB has been used to identify a new class of glutamate receptors which shows a different pharmacological profile than the 3 classes of glutamate receptors characterized by Quis, NMDA and KA. (Fagg et al.,1982; Mena et al.,1982). These authors have shown that whilst APV is the most effective of the phosphono-analogues at inhibiting glutamate binding in the absence of added ions (Ca^{2+} and Cl^{-}), this population of receptors accounts for only 20% of the total binding in the presence of these ions. The 80% of binding sites for

glutamate that are dependent on the presence of Ca^{2+} and Cl^- are, in contrast, much more strongly inhibited by APB. Significantly the binding of L-aspartate was not affected by any of these substances, either in the presence or absence of $CaCl_2$. There is strong evidence based on pharmacological studies that these Ca/Cl dependent APB sensitive glutamate binding sites in purified SPM's are the same as the receptors mediating synaptic transmission in the hippocampus. (Koerner et al., 1982). In contrast, the inhibition of the synaptic response in the spinal cord, which is mediated by an NMDA type receptor, has a pharmacology much more similar to the Ca/Cl^- independent receptors, where APV is the more powerful antagonist.

Thus, a thorough and exhaustive investigation of binding sites at all stages during ontogeny using these compounds in particular, together with the natural amino acid ligands L-Asp, L-Glu and homocysteate, may allow a description of the process of development and specification of aminoacidergic transmitter systems.

Another approach to the characterization of amino acid receptors could be a classification in terms of the ability of certain cations to affect the binding of either aspartate or glutamate. The divalent cation Mg^{2+} was one of the first agents that was found to discriminate between different amino acid excitants. Low concentrations of Mg^{2+} depressed responses to NMDA in the frog spinal cord whilst having little or no effect as responses to L-Glu, Quis, or KA (Evans et al, 1977). Co^{2+} and Ni^{2+} were even more potent than Mg^{2+} in exhibiting the same effects, whilst Mn^{2+} was less potent than Mg^{2+}. The monovalent cations K^+, Li^+, Cs^+ and Rb^+ all produced a dose dependent inhibition of glutamate binding in the cerebellum (Roberts and Sharif, 1981), and

in the hippocampus (Baudry and Lynch, 1980). In addition, these ions had a similar effect on aspartate binding in the cerebellum (Roberts and Sharif, 1981). It is difficult to say exactly what the role of these cation effects are; they may be part of a regulatory mechanism controlling either the affinity or the numbers of the binding sites. If this is so, then these ions may have different effects on glutamate and aspartate sites at different stages in ontogeny and may help to regulate the developmental process, even though differences in their effects in the adult are not that apparent. Na^+ and Ca^{2+} however, do show differences in their effect on glutamate and aspartate binding. Foster et al. (1981) have demonstrated that L-Asp binding is increased 4-fold to rat forebrain membranes in the presence of Na^+, whilst glutamate binding is unaffected by Na^+. Although Na^+ dependent binding may in some cases be synonymous with binding to a re-uptake site, this would also effect glutamate as the two dicarboxylic amino acids share the same high affinity uptake site. In the light of this, the authors suggest that the effect of Na^+ on aspartate binding is due to a regulatory mechanism on the receptor site. Although the Na^+ dependent binding of glutamate and aspartate in the cortex does seem to be related to an uptake site, a full study of the dose dependent effect of Na^+ at various age points may prove interesting.

The greatest difference in effect on glutamate and aspartate binding is exhibited by Ca^{2+}. In rat forebrain, a Ca^{2+} concentration of 2.5mM resulted in a 3.5 fold increase in L-Glu binding while L-Asp binding was unaffected. (Foster et al, 1981). An exactly analogous situation was found in hippocampus (Vargas and Costa, 1981). In all cases, Ca^{2+} evokes an increase in the number (B MAX) of binding sites

whilst their affinity remains constant. Baudry and Lynch (1980) were the first to suggest, on the basis of their experiments, that the effect of Ca^{2+} is to activate endogenous membrane-associated thiol proteases which then uncover additional glutamate receptors. In contrast, on the basis of pharmacological evidence, Foster et al.(1981) suggest that, in the presence of Ca^{2+}, ^{3}H-L-Glu labels a different population of receptors than in its absence.

The interesting point of this work, though, is that these effects of Ca^{2+} are present in some brain regions and not in others. In the cerebellum, Ca^{2+} produces an enhancement of binding at low concentrations, but an inhibition at concentrations above 5mM, and has the same effect on glutamate and aspartate. Baudry and Lynch (1980) have further suggested that Ca^{2+} dependent proteases may be responsible for the long term potentiation of synaptic transmission found in hippocampal synapses after brief periods of high frequency stimulation, and that it is therefore glutamate receptors that are responsible for this long term synaptic facilitation. If this theory is correct, then it may be that the effects of Ca^{2+} would only be expected in areas that need to possess a high degree of synaptic plasticity such as the cerebral cortex, but not in essentially hard-wired circuits such as the cerebellum.

It would, therefore, be extremely interesting to investigate the effect of Ca^{2+} on glutamate and aspartate binding throughout ontogeny in the cortex, and to relate any effect to neurophysiological studies charting the development of L.T.P. The effects of freeze thawing reported in Section 5.6 show that there is indeed a population of glutamate receptors that are normally hidden, although hidden aspartate receptors do not exist.

In addition to any effect of Ca^{2+} in the activation of proteases, this ion has recently been shown to enhance glutamate binding that is stimulated by the presence of Cl^- ions. (Fagg et al 1982). Unlike previous studies reported earlier (e.g. Foster et al.,1981), these later studies demonstrated that Cl^- ion was an absolute requirement for Ca^{2+} stimulation, as Ca^{2+} alone did not affect glutamate binding (Whittemore et al.,1983). The explanation for the difference in those results probably resides in the fact that the earlier binding studies were carried out in 50mM Tris/HCl buffer so that the concentration of Cl^- was sufficient to allow expression of the Ca^{2+}/Cl^- dependent sites. This work has two exciting implications, as previously mentioned. The first is that Ca^{2+} or Cl^- ions have no enhancing effect on aspartate binding, so it may be that in their presence, only one particular type of amino acid receptor is being measured. Secondly, unlike the Ca^{2+}/Cl^- independent sites, the ion dependent sites are potently antagonized by the glutamate analogue APB. The pharmacological requirements of this site, explored by displacement studies with a range of amino acid agonists and antagonists, exactly parellels the pattern of the pharmacology of inhibition by APB of several neuronal pathways (see Whittemore et al.,1983). So these Ca^{2+}/Cl^- dependent, APB sensitive, Na^+ independent sites are strongly implicated as postsynaptic receptors mediating transmission in the CNS. Again, it would be valuable to use these discriminatory ions in binding studies throughout development.

There is increasing evidence from many <u>in vivo</u> studies that cGMP may be the second messenger involved in the translation of the initial transmitter/receptor interaction to the physiological response. In addition, it has been shown, using cerebellar slices and cell suspensions, that different amino acid agonists vary in their ability to stimulate

cGMP production (Schmidt et al, 1975; Garthwaite and Balazs, 1981). As a corollary of this, Sharif and Roberts (1980) have demonstrated that guanine nucleotides produce a substantial inhibition of ^{3}H-glutamate binding and that this effect was not observed with adenine nucleotides. Thus, guanine nucleotides may be regulating receptor sensitivity by their effects on a mechanism that is also directly linked to the production of the physiological effect. The concept of a receptor modifying protein, the G protein, sensitive to the guanine nucleotides, was further discussed in Chapter 2. If the ability of GTP to affect amino acid binding is indicative of the presence of <u>functional</u> amino acid receptors, then a study of the effect of these nucleotides on different types of amino acid binding sites at different stages in development may give some insight into when exactly such binding sites become functional.

All of these studies may be more fruitful if they were carried out using cell membranes from cortical areas that were known to be rich in glutamatergic or aspartergic nerve endings. It may be possible to identify such projection pathways into the cortex by using sensory deprivation, chemical and surgical lesioning techniques and congenital mutants, and correlating the effects of such techniques with changes in biochemical parameters relevant to glutamate or aspartate transmitter systems.

One of the long term aims of neurochemical studies designed to characterise physiologically relevant, synaptically active binding sites for neurotransmitters must be to isolate and characterize the receptor complex itself, and to describe its chemical nature. The first step in such studies would be to probe the relationship between

the receptor complex and its membrane environment, and the structure of the receptor molecule itself. This could be achieved by an examination of the effects of protein and membrane modifying agents on amino acid binding. Similar work carried out on glutamate receptors in the cerebellum (Sharif and Roberts, 1980), and in whole brain (Michaelis, 1975; Michaelis, 1979; Michaelis et al, 1980), have led to the suggestion that the glutamate receptor may be a metallo-glycoprotein with an iron sulphur (Fe_2S_2) at its centre. It would be very interesting to use these methods to investigate the nature of glutamate and aspartate binding sites in the cortex, and, given my prime interest in development, to apply the techniques to a study of the amino acid receptors throughout development. This approach may shed some light on the problem of how, and when, receptor complexes reach maturity and become stabilized in their lipid environments.

Again in the long term, pharmacological agents may emerge with specific affinity for amino acid receptor sites at different developmental stages. These agents could then be used to label receptor proteins which may then be directly visualized by the application of gel electrophoresis. As the glutamate receptor is a glycoprotein, lectins may also provide a useful label. If receptor complexes can be separated from other membrane components, then the possibility arises of raising antibodies to these receptors, which would provide a very specific marker for visualization of these binding sites throughout the process of development.

These then are the main directions in which the work reported in this thesis could proceed. These approaches also demand an integration of research across related disciplines. Neurophysiological, neuro-

anatomical and immunological techniques all need to be used, alongside classic neurochemical studies. I believe a more multi-disciplinary approach to the problem of how neurotransmission is mediated in the CNS, and the factors governing the development of such systems, is not only desirable, but essential. The implications of an increased knowledge in this area are enormous. My work, and studies by many other groups, make the case for involvement of glutamate and aspartate in neurotransmission in the CNS very strong indeed. In addition, the glutamatergic and aspartergic systems may provide an excellent model for the investigation of the processes by which specification of pathways is brought about.

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